# IDENTIFICATION AND CHARACTERIZATION OF TYPE III SECRETION INHIBITORS IN ERWINIA AMYLOVORA, THE CAUSAL AGENT OF FIRE BLIGHT OF APPLE AND PEAR

BY

FAN YANG

THESIS

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Adviser:

Assistant Professor Frank (Youfu) Zhao

**ABSTRACT**

Fire blight is a destructive bacterial disease of apples and pears as well as other rosaceous plants and causes millions of dollar losses around the world each year. *Erwinia amylovora* is the causative agent of fire blight. The type III secretion system (T3SS) and exopolysaccharide (EPS) amylovoran are two major yet separate virulence factors in *E. amylovora*. Current fire blight management mainly relies on application of copper compounds and antibiotics. However, development of streptomycin resistant *E. amylovora* isolates in the US and other countries has made it ineffective. It is reasonable to believe that disabling the T3SS function may provide another way of controlling bacterial diseases. High-throughput screening of chemical libraries have identified small molecule inhibitors that attenuate T3SS of mammalian pathogens, but no study has been reported so far for plant pathogenic bacteria. In this study, three small molecules were identified to delay hypersensitive response (HR) development in tobacco. Using GFP as a reporter, six chemicals were identified to suppress T3SS gene expression of *E. amylovora* under *hrp* inducing conditions. Five of them belong to salicylidene acylhydrazides. Among them, chemical # 3 and # 9 were most potent in inhibiting T3SS gene expression in a dose-dependent manner without affecting bacterial growth. Furthermore, this group of chemicals also inhibited exopolysaccharide amylovoran production. Our results further demonstrated that chemical # 3 treatment resulted in growth reduction and symptom suppression on crab apple blossom. To better understand the mode of action of this class of compounds, we carried out a microarray analysis of *E. amylovora* treated with chemicals # 3 and # 9. A total of 534 and 183 genes were identified to be significantly differentially regulated by chemicals # 3 and # 9 treatment, respectively. The majority of genes in *E. amylovora* T3SS cluster including *hrpL* as well as effectors including *avrRpt2* and *hopC1* were down-regulated more than two folds by both chemicals # 3 and # 9. Chemical # 3 also suppressed the transcription of all amylovoran biosynthesis genes. Interestingly, the most significant upregulated genes were those involving iron acquisition and utilization. In addition, other compounds of this group (chemicals # 1, # 2, # 4) also exhibited a similar effect on gene expression, i. e suppressing T3SS and *ams* gene expression, while promoting iron uptake gene expression. Our results suggested that

a common inhibition mechanism may be shared by these compounds. To test whether salicylidene acylhydrazides affect secretion of T3SS proteins, total secreted proteins from wild type bacteria grown in the presence or absence of chemicals # 3 or # 9 were quantified. Our results showed that chemical # 9 exhibited a dose-dependent inhibition of protein secretion in *E. amylovora*; while chemical # 3 was more effective, causing a complete blockage of secretion of T3SS proteins at as low as 5 μmol.

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# LITERATURE REVIEW

## 1.1 Fire blight disease and symptoms

Fire blight, caused by the Gram-negative bacterium *E. amylovora*, is a serious bacterial disease in Rosaceae family plants (Zhao *et al*., 2005). It mainly infects three economically important pome fruit trees, apple (*Malus sylvestris*), pear (*Pyrus communis* L.) and quince (*Cydonia oblonga* Mill). Fire blight was also the first plant disease known to be caused by a bacterium. It was first named by Willian Coxe in 1817, because its symptom resembles the appearance as if the plant had been burnt by a hot flame. In 1878, Thomas J. Burrill observed living and moving atoms in the discolored plant tissue affected by fire blight under the microscope, and proposed that bacteria might be the cause of this disease. In1884, T. J. Burrill's bacteria theory was demonstrated by Joseph Charles Arthur. He carried out several cross-inoculation experiments, in which isolated bacteria directly caused blight symptom yet bacteria-free filtration did not (van der Zwet *et al*., 2011).

The characteristic symptoms of fire blight can be easily recognized with the appearance of blackening tissue as though they had been scorched by fire (Schroth *et al*., 1974). After blossoms turn brownish or black following disease establishment, infection can progress rapidly along shoots and twigs, resulting in ooze drop or cobweb-like bacterial stand under wet conditions. Shoot blight also forms typical shepherd's crook symptoms at wilting tips, in which blackening of the leaf midrib is often present. Fruit blight is associated with prematurely red or green margins along the necrotic area. Finally, infection may spread downward into larger limbs or trunk, in which canker extension kills part of limb or the whole tree (van der Zwet *et al*., 2011).

The first report of fire blight on apple, pear and quince came from Hudson River Valley in New York in 1780. Soon after, this disease became prevalent in the U.S. and spread into other countries, such as New Zealand and the United Kingdom. Over the past century, fire blight has spread around the world, including North America, west Pacific region, Europe and Mediterranean area (Bonn and van der Zwet, 2000). Although its wide dissemination has been attributed to human activities involved in long distance

moving, fire blight is more likely to outbreak in the areas with humid and wet weather condition in the presence of susceptible host plant.

Fire blight poses multiple threats to the global pome fruit industry. Blossom infection and twig blight can cause a strong decrease in current crop yield and that of next growing seasons. In susceptible hosts, infection may spread from trunk into limbs and result in death of entire tree. Fire blight causes about $ 100 million of dollar losses in the U.S.A. each year. This value has become increasingly higher in many production regions because of susceptible apple cultivar and favorable climate condition. Fire blight also causes great financial loss in major apple and pear production areas outside the U.S. For example, the Swiss government reported a $27.5 million dollars of loss due to fire blight outbreak in 2007 alone (van der Zwet *et al*., 2011).

### 1.2 *E. amylovora*

*E. amylovora* is the causative agent of fire blight, a short rod filamentous Gram-negative bacterium. Several other related *Erwinia* species have also been reported, including *E. pyrifoliae*, *E. tasmaniensis* and *E. billingiae*. Comparative genome sequencing analysis, both from pathogenic and non-pathogenic *Erwinia* species, provides new insights into evolutionary relationship, virulence divergence and host specificity within this genus. For example, *E. amylovora* strain CFBP1430 was isolated from *Crataegus* in France in 1972; whereas *E. amylovora* strain Ea273 was from New York, also known as ATCC 49946; both have a wide host range (Buban *et al*., 2003). A large-scale chromosomal rearrangement is apparent in the comparative genomic analysis of the two strains though both share more than 99.99% identity on nucleotide level (Smits *et al*., 2011). Moreover, the genomes of *E. pyrifoliae* strains, isolated from South Korea and Japan, are almost identical. However, absence of several characteristic virulence-associated traits in *E. pyrifoliae*, including genes encoding levansucrase, protease A and effectors, might account for its limited host range (Zhao and Qi, 2011). Two saprophytic microorganisms, *E. tasmaniensis* and *E. billingiae*, show significantly distant genetic composition compared to pathogenic relatives.

### 1.2.1 Type III secretion system

*E. amylovora* belongs to the family *Enterobacteriaceae*, which also includes genera *Escherichia*, *Pantoea*, *Salmonella*, *Shigella* and *Yersinia*. This group of pathogens shares a similar mechanism to deliver effector proteins into their eukaryotic host cells (Büttner and He, 2009). The process of effector secretion and translocation is achieved by the type III secretion system (T3SS) (Cornelis, 2006). Different species secrete distinct combinations of effector proteins, ranging from symbiosis establishment to pathogenic effects. T3SS is a structurally and functionally conserved macromolecular machine. T3SS consists of secreted proteins, chaperones and cytoplasmic regulators, as well as a needle apparatus. The apparatus is made up of three structural components: an inner-outer membrane ring, a protruding extracellular needle complex and a pore-forming translocon (Izoré *et al*., 2011).

Pathogens have evolved into precise regulatory mechanism to build up functional T3SS. In the early stage of assembly, needle subunit proteins are exported that form an increasing hollow channel. The bacterium switches exported substrate of the apparatus to regulate needle length by assembling the tip protein into the needle end when it contacts host cell. The secretion of effectors is always blocked until the gatekeep protein perceives the presence of the host plant (Deane *et al*., 2010). Poyraz *et al*. (2010) generated a functional mutant of potomer, a tiny protein consisting of needle component in *Shigella flexneri* and *Salmonella typhimurium*. It was found that protomer experienced a conformation change from α-helix into β-strand in ATP-independent manner during needle assembly. A pathogenic bacterium prevents the premature secretion of effector before contacting the plant host. In a recent study, *mxiC* mutant strain constitutively secreted effector in the absence of the host plant, indicating that MxiC positively regulates secretion of translocator protein and inhibits release of effectors. MxiC is a cytoplasmic protein that might perceive the host signal through needle complex (Martinez-Argudo *et al.* 2010).

### 1.2.2 Type III secretion system is a key virulence factor of *E. amylovora*

In *E. amylovora*, T3SS is encoded by the *hrp* gene cluster, which is required to elicit a hypersensitive response (HR) on non-host plants and cause disease on susceptible

host plants. The *hrp* gene cluster is located on a 62 kb genomic DNA pathogenicity island 1 (PAI-1). In addition to *hrp* gene cluster, PAI-1 also contains the effectors and elicitors (HEE) region, Hrp-associated enzymes (HAE) region and island transfer (IT) region (Zwet *et al*., 2011).T3SS of *E. amylovora* contains 27 genes, encoding two harpin proteins (HrpN and HrpW), several effectors, four regulatory proteins (HrpL, HrpS and HrpXY) and other structural component proteins. Harpin protein HrpN plays an essential role in virulence and avirulence activities of *E. amylovora*. Wei *et al.* (1992) first purified the harpin protein from *E. amylovora* and found it was required to trigger HR when infiltrated into tobacco, tomato and *Arabidopsis thaliana*. The *hrpN* mutant was incapable of causing disease in pear or eliciting a HR. A later study found that the C-terminal half of the protein was required for its virulence and avirulence activities, and was highly conserved among many plant pathogenic bacteria (Sinn *et al*., 2008). Recent evidence suggests that HrpN protein might facilitate the translocation process of its own and other effectors when *E. amylovora* delivers a suite of effector proteins (Boureau *et al*., 2011). HrpW contains a pectate-lyase domain homologous with type 3 pectatelyase, and partially participates in callose deposition. *DspA/E*, homologous with *avrE* of *Pseudomonas syringae* pv. *tomato*, encodes a virulence factor of *E. amylovora* (Bogdanove *et al*.,1998; Gaudriault *et al*., 1997). Besides, *E. amylovora* contains other effectors, including EopB and AvrRpt2. Histidine kinase HrpX and response regulator HrpY consist of two component signal transduction system, which senses environment stimuli and regulates T3SS function. HrpL is a master regulator that controls T3SS apparatus assembly and effector synthesis by recognizing HrpL-dependent genes. In turn, HrpL is known to be regulated by HrpS at transcriptional level (McNally *et al*., 2011). The majority of remaining genes in the *hrp/dsp* cluster encodes components of T3SS macromolecular machine. For example, *hrpA* encode Hrp pilus in *E. amylovora*.

### 1.2.3 EPS: a different virulence factor

Exopolysaccharide (EPS) produced by *E. amylovora* plays important roles in disease establishment and dissemination. Accumulation of EPS often results in water flow disruption of plant tissue, followed by ooze exudation on plant surface. *E. amylovora* produces two types of EPS, levan and amylovoran. Levan, a homopolymer of

fructose residues resulting from sucrose cleavage by levansucrase, has been reported in many plant pathogenic bacteria (Gross *et al*., 1992). In contrast, amylovoran is a complex acidic heterpolysaccharide, consisting of galactose, glucose and pyruvate residues (Nimtz *et al*., 1996). The *ams* gene cluster is responsible for amylovoran biosynthesis, which is strictly regulated by the Rcs phosphorelay system (Wang *et al*., 2012; Zhao *et al*., 2009). Mutant strains impairing in their ability to synthesize amylovoran were completely nonpathogenic on apple trees or immature pear fruits (Wang *et al*., 2012).A recent report has established a close connection between EPS production and biofilm formation. Biofilm formation was disrupted in *ams* and *lsc* mutants of *E. amylovora*, indicating an essential role biofilm plays in its pathogenesis (Koczan *et al*., 2009).

### 1.3 Fire blight management

Host resistance to fire blight is a desirable property in pear and apple breeding. The first pear breeding program was initiated in Iowa in 1867. At that time, people attempted to produce hybrid cultivars with disease resistance as well as superior fruit quality. The five most economically important *Pyrus* species exhibit a wide range of resistance against fire blight. However, no commercial pear cultivars introduced from Europe are known to grow well where weather conditions favor *E. amylovora* growth. In 1940s, the USDA started a series of intercrossing experiments by testing selective cultivars of several breeding programs. Later, a variety of native germplasms were collected around the world for the genetic base of a new breeding program. All cultivars released so far have been proven to have very limited resistance to fire blight. Although fire blight is usually less severe in apple than in pear, it still causes significant losses in many commercial apple cultivars, such as Jonathan, Gala, Fuji and others. The apple breeding program also went back 100 years in the USA. Unfortunately, there is no truly resistant apple cultivar available (van der Zwet *et al*., 2011).

A few reports have shown that plant growth regulators (PGR) induce host resistance to *E. amylovora* in pear and apple. Goodman and Hemphill (1951) found that indole-3-acetic acid (100 mg · $L^{-1}$) reduced disease occurrence in apple trees by about 52%. Klos *et al.* (1969) reported that treatment of daminozide (1,000 mg · $L^{-1}$) decreased in the number of infected pear shoots by 61%. In the 1990s, several papers reported that

the gibberellin biosynthesis growth inhibitor prohexadione-calcium (P-Ca) decreased fire blight incidence. Later, air-blast sprays in apple orchards proved that P-Ca was effective in controlling fire blight by reducing shoot tip infection compared to that of untreated-trees. In addition, the P-Ca was shown to protect secondary blooms of pear from *E. amylovora* infection. The precise mechanism of the action of P-Ca remains unclear (van der Zwet *et al*., 2011).Some studies suggest that P-Ca treatment not only interferes with *E. amylovora* motility in leaf xylem and parenchyma tissue, but also alters flavonoid metabolism and cell wall width (Spinelli *et al* 2006; McGrath *et al*., 2009).

Pruning is useful to remove infected plant tissue in the early growing season. Canker and other dead tissue that *E. amylovora* overwinters need to be removed. It is better to cut off all infected sections as well as nearby health tissue by using disinfested pruning tools.

Current fire blight management mainly relies on application of copper compounds and antibiotics. They are usually sprayed prior to or during blossom for the purpose of reducing or eliminating early infection by *E. amylovora.* Copper sulfate mixed with lime (Bordeaux mixture) has been the most commonly used because of its low cost and excellent protection. Other copper compounds have been proven to be effective in controlling fire blight, but they are seldom used in orchards due to potential fruit russet. Cytotoxicity is the major health concern for all copper compounds. Cupric ions are released when plants and bacteria convert copper into a soluble formation, which is highly toxic to humans and animals. Streptomycin belongs to a large family of compounds known as aminoglycosides. This class of drugs cause cell death by incorporating inappropriate amino acids into elongating peptide strand (Kohanski *et al.*, 2010). Recent evidence suggests that this class of drugs also alter membrane integrity of Gram-negative bacteria, resulting in increasing uptake of the drug. Streptomycin was first proven to reduce incidence of blossom blight on Jonathan apple by Murneek in 1950s. Since then, it has become the most potent chemical agent in controlling fire blight in apple and pear orchards (van der Zwet *et al*., 2011). The antibiotics are generally the most potent among other control measures, in which the overall mean disease control ranged from 59.8 to 61.7 %. Streptomycin exhibited the best performance, yielding a 68.6 % of mean disease control, while the value of copper products reached 37.6 %.

Among the biological controls, *Bacillus subtilis* showed the highest mean disease control (31.9 %), followed by *Pantoea agglomerans* (25.7 %). Interestingly, none of plant defense-activating compounds produced a satisfactory disease control value.

Development of streptomycin resistant (SmR) *E. amylovora* isolates in the US and other countries has made streptomycin ineffective in fire blight management. The first SmR strain was isolated from pear orchards in California in 1971(Miller and Schroth 1972). Later, streptomycin resistant *E. amylovora* isolates were found in Washington, New York, Oregon, Missouri, Michigan and other countries (Russo *et al.*, 2008; Loper *et al*., 1991; Coyier and Covey, 1975; Shaffer and Goodman, 1985; Manulis *et al*., 1998; Norelli *et al*. 2003; McGhee and Sundin, 2011).Therefore, developing new antibacterial agents for prevention or treatment of fire blight becomes necessary. McGhee and Sundin (2011) evaluated the effectiveness of kasumin in controlling fire blight. Kasumin is an aminoglycoside antibiotic that interferes with bacterial protein synthesis. It was found that both kasumin and streptomycin led to a 100 fold decrease in *E. amylovora* population compared to no treatment when bacteria were inoculated on apple blossoms 24 h prior to chemical application. When antibiotics and a bacterial suspension were simultaneously applied to apple blossom, kasumin resulted in about a 30 fold reduction in bacterial population compared to water treatment 24 h post inoculation. Interestingly, they observed no kasumin sensitivity in other bacterial species. These findings suggested that kasumin was a potential antibiotic for fire blight management.

### 1.4 Antimicrobial drugs targeting T3SS

Increasing prevalence of antibiotic resistant strains pose a great challenge for clinic treatment and agricultural management. It is believed that resistant occurrence is the result of bacterial adaptation in response to harmful cellular perturbations induced by antibiotics. Thus, it is reasonable to assume that a weak selection pressure imposed by chemotherapy upon bacterial populations would diminish or slow down resistance development. Despite extensive investigation of drug-target interaction and drug molecule modification, researchers are now only beginning to scratch the surface in developing antibacterial agents with a novel mechanism targeting bacterial virulence factors. The ability of pathogenic bacteria to thrive in host environments and to establish

infection has been widely attributed to a range of virulence factors or regulators, such as T3SS, global regulator, adhesion and invasion protein, quorum sensing, two-component regulatory system and so on (Alksne and Projan, 2000; Keyser *et al*., 2008).

To identify virulence blockers against the cholera pathogen *Vibrio cholera*, Hung *et al*. (2005) carried out a high-throughput screen of a 50,000-compound small molecule library. They constructed a virulence based reporter system in which the tetracycline resistance gene (tetA) was controlled by the cholera toxin (ctx) promoter. A small molecule virstatin was found to inhibit cholera toxin (CT) production under *in vitro* inducing conditions. At 50 μmol, virstatin suppressed gene expression of co-regulated pilus (TCP) and CT via direct inhibition of AraC-like transcriptional activator ToxT. In addition, *V. cholera* colonization on small intestines of infant mice was reduced in the presence of virstatin. The reduced colonization was only observed in the TCP-dependent strain, suggesting that virstatin functions specifically on ToxT.

A recent report identified a small molecule that specifically targets RNA degradation in Gram-positive pathogenic *Staphylococcus aureus* (Olsen *et al.*, 2011). It is clear that ribonuclease RnpA plays an important role in controlling virulence gene expression via mRNA turnover. In following chemical screening, compound RNPA1000 was identified to interfere with RnpA-mediated RNA degradation process *in vitro*. RNPA1000 exhibited a significant antimicrobial activity against several multi-drug resistant strains, such as *S. pneumoniae, S. pyogenes* and *S. agalactiae* (Olsen *et al.*, 2011).

T3SS has become a logical target for chemotherapeutic intervention, because its homologous macromolecular apparatus has been identified only in Gram-negative bacteria and is a key virulence determinant in many plant and mammalian pathogens (Baron, 2010). To identify compounds specifically blocking T3SS function, many laboratories conducted chemical screening that was centered on custom cell assays targeting known T3SS processes, including gene expression, effector secretion and translocation, and symptom development. These screens have led to the identification of several classes of T3SS-inhibiting compounds.

In 2003, Kauppi *et al*. (2003) first constructed a screening strain of *Y. pseudotuberculosis* that carries luciferase-encoding hybrid gene *luxAB* under the control

of effector *yopE* promoter. From a 9,400-compound library, about 30 compounds were found to decrease luciferase light signal by at least 40%. Excluding compounds that affect bacteria growth, three compounds were chosen for further characterization. It was found that compound # 2 to 4 inhibited YopE, YopD and YopH secretion of *Y. pseudotuberculosis*. Compound 2, which belongs to a class of acylated salicylaldehyde hydrazones, exhibited a dose-dependent inhibitory effect on bacterial motility as well as luciferase light emission *in vitro*. Interestingly, compound 3 salicyanilide interfered with Yop secretion without affecting flagellum assembly and motility. Compound 4 was less effective in inhibiting Yop secretion. Structural-function relationship assay suggested that many compounds from salicylidene acylhydrazide family shared significant biological activities against T3SS in *Yersinia*. It was found that compound 1 specifically interfered with *Yersinia* outer protein secretion without affecting Yop expression or autoregulatory transcription activator LcrF. Besides, cell-associated bacteria were significantly reduced when *Y. pseudotuberculosis* infected HeLa cells in the presence of compound 1, which was due to blockage of YopH translocation. In addition, another 15 salicylidene acylhydrazides were found to inhibit YopE secretion *in vivo* (Nordfelthn *et al*., 2005). Later, they developed new strategies in identifying T3SS inhibitors through analogue synthesis and biological effect evaluation. It was found that hydroxyl or acetylester group was required for inhibitory ability of this family compounds, and that replacement of iodine at position 3 into hydrogen would improve their activity (Dahlgren *et al*., 2007). A number of compound 1analogous were synthesized and underwent the same biological activity assay (Kauppi *et al*., 2007).

Later publications have shown that this family of compounds broadly interferes with T3SS function in a number of animal pathogenic bacteria. The obligate intracellular pathogen *C. trachomatis* injects a number of effector proteins into inclusion membrane or host cytosol through T3SS. It was found that INP0400 reduced the number of intracellular *C. trachomatis* and the size of the inclusion bodies when bacteria infected McCoy cell, suggesting this compound inhibited *C. trachomatis* infection. This compound also disrupted effector translocation and homotypic fusion, which is required for Chlamydia infection (Muschiol *et al*., 2006).INP0341 is more effective in inhibiting *C. trachomatis* L2 replication than INP0400 does *in vitro* (Muschiol *et al*., 2009). Recent

evident suggests that INP0341has the ability to protect mice from a *C. trachomatis* vaginal infection by reducing inclusion-forming units (Slepenkin *et al.*, 2011). In addition, N'-(3,5-dibromo-2hydroxybenzylidene)-4-nitrobenzohydrazide (C1) suppresses the infectivity of *C. trachomatis* on HeLa cells in a dose-dependent manner (Wolf *et al*., 2006).

In *E. coli*, a group of salicylidene acylhydrazides inhibit effector secretion *in vitro*. Microarray analysis revealed that transcription of genes in SOS response and DNA damage were not induced by the compounds, which was in agreement with the observation of no effects on bacterial growth. Interestingly, these compounds consistently suppressed expression of all genes that were located on the locus of enterocyte effacement (LEE) pathogenicity island, as well as that of non- LEE effectors (Tree *et al*.,2009).

Other studies revealed that salicylidene acylhydrazides have the ability to disrupt needle assembly of *Shigella flexneri*. It was found that INP0401 and INP0402 prevented *S. flexneri* from invading HeLa cells and disrupted the T3SS-mediated macrophage apoptosis. These compounds did not affect protein levels of needle subunit MxiJ and MxiH. However, the proportion of incomplete needle structure was significantly increased after chemical treatment, indicating that this family compounds disrupted T3SS of *Shigella flexneri* likely through suppression of secreton assembly (Veenendaal *et al*., 2009).

Similar inhibitory effects were observed in *Salmonella enteric* serovar Typhimurium. There are two T3SSs in *Salmonella*. T3SS-1, located on *Salmonella* pathogenicity island (SPI)-1, plays a significant role in early stage of infection by promoting bacteria entry into intestinal epithelia. T3SS-2, encoded by SPI-2, is required for its intracellular survival. INP 0007 and INP 0403 inhibited secretion of T3SS-1 effector proteins in a dose-dependent manner. The two compounds not only disrupted Sip-mediated pore-forming activity in *S. typhimurium*, but also reduced the magnitude of the secretory and inflammatory responses compared to that of DMSO treatment (Hudson *et al*., 2007).Moreover, nine different salicylidene acylhydrazides (D1- D9)were found to disrupt effector secretion of *S. typhimurium* and its ability in invading epithelial MDCK cells (Negrea *et al*., 2007). Layton *et al*. (2010) defined the genome-wide transcriptional

perturbation of *S. typhimurium* mediated by INP0403 under T3SS-1-inducing condition. The compound reduced the transcript of genes encoding the T3SS-1 structural apparatus, regulators and chaperones. The transcription of four positive regulators of SPI-1 were significantly reduced, all of which are AraC-like transcriptional activators. It was also found that few T3SS-2 genes were differentially expressed in the microarray analysis. Interestingly, about one quarter of genes significantly changed after INP0403 treatment were those involving iron acquisition system. These included *tonB* and its dependent receptors (*exbBD*), ABC ferric transporter (*fepABCDEG*), iron transport proteins (*feoAB, fhuABCDEF*), iron uptake regulator (*fur*) and other iron related genes. When adding exogenous iron (ferrous or ferric form), INP0403-mediated inhibitory effect on secretion of T3SS-1 protein was prevented. Thus, it is possible that inhibition of T3SS gene expression and protein secretion in *Salmonella* is due to iron depletion mediated by INP0403.

Other classes of small molecule inhibitors have also been reported. For example, compound 1 (2-imino-5-arylidene thiazolidinone) is known to target T3SS protein secretion of *S.typhimurium* and *Y. enterocolitica*, respectively. Proteins that consist of needle complex (InvG, PrgH, PrgK) were decreased when wild type *S. typhimurium* was grown in the presence of compound 1, suggesting this compound target needle complex formation. Besides, this chemical has the ability to interfere with functions of T2SS in *P. aeruginosa* and T4SS pili secretion system in *Francisella novicida*. Most strikingly, compound 1 also prevented HR when *P. syringae* is co-inoculated on non-host tobacco, suggesting it could inhibit T3SS in plant pathogenic bacteria (Felise *et al*., 2008). However, compound 1 is known to have poor solubility and active only in high micromolar concentrations. Later reports illustrated that novel solid phase synthesis approach and structure modification are useful to improve bioactivity and physiochemical properties of thiazolidinone compounds (Kline *et al*., 2008& 2009).

Three chemicals are known to exhibit inhibitory effects on secretion of T3SS substrates of *Y. pestis* (YopH, YopM and YopD). These chemicals have no effects on bacterial growth or HeLa cell viability. Moreover, these compounds had the ability to inhibit effector delivery in *Y. pestis* and enteropathogenic *E. coli* (EPEC), respectively.

Interestingly, these compounds had different structures and differed from other known T3SS inhibitors (Pan *et al*., 2009).

Aiello *et al*. (2010) designed two high-throughput screens to identify small molecules that would disrupt T3SS function of *P. aeruginosa*. Compound 1was identified to reduce effector transcription and secretion without affecting bacterial growth. It was shown that compound 1blockedeffector ExoU translocation when bacteria infected CHO cells in a concentration-dependent manner, which was consistent with the finding on another known ExoU inhibitor pseudolipasin.

Six novel compounds were found to block effector translocation of *Y. pseudotuberculosis* into mammalian cells (Harmon *et al*., 2010). Abundant effector YopE leakage was observed in the culture supernatant instead of host cell after chemical treatment. Interestingly, the inhibition was not due to disruption of T3SS assembly or effector secretion. Moreover, five chemicals also blocked the translocation of ExoS, a T3SS effector from *P. aeruginosa,* into HEp-2 cell.

Recently, a linear polyketide compound aurodox was found to strongly inhibit bacteria- induced hemolysis without affecting EPEC growth (Kimura *et al.,* 2011). Aurodox reduced T3SS-dependent protein secretion in a dose-dependent manner under *in vitro* condition. It was also found that oral administration of aurodox contributed to the survival of mice that had received a lethal dose of *Citrobacter rodentium*. This was the first evidence that TTSS inhibitor could be used as anti-infective agent on animal model.

However, the precise molecular mechanism of inhibition by small molecules remains unknown. Up to now, only two reports have claimed that they have determined the cellular targets for a few compounds. It is clear that mutant strain disrupting *lcrF*, a transcription factor regulating expression of *Yersinia* T3SS, caused an attenuated infectivity both in tissue culture and animal infection models. Researchers thus screened over 250 benzimidazole compounds for inhibiting LcrF binding ability on its known promoter binding sites. Two compounds were found to considerably reduceT3SS-mediated cytotoxicity toward macrophages and also protect mice from *Y. pseudotuberculosis* infection in lungs (Garrity-Ryan *et al*., 2010). Most recently, Wang *et al*. (2011) employed affinity chromatography method to identify putative target proteins of salicylidene acylhydrazide in *E. coli* O157. Three proteins (Tpx, WrbA and FolX)

were isolated using Affi-Gel-labeled compound from cell lysate. Microarray analysis was performed to compare gene expression in *tpx*, *wrbA* and *folX* mutants with those of *E. coli* O157 WT strain. Combined with previous results, transcript levels of genes, which are suppressed in response to compounds (ME0052-0054), were up-regulated in mutant strains, suggesting that these three proteins could potentially be the targets of the compounds. Thiol peroxidase (Tpx), 2-Cys peroxiredoxin, has been implicated as a mediator of defense response against ROS in many pathogenic bacteria. It covalently binds to ROS and releases $H_2O$, leading to formation of a disulphide bridge between cysteine residues (C61 and C95) (Baker and Poole, 2003). A recent study solved the structure of Tpx from *Y. pseudotuberculosis* in the oxidised and reduced states (Gabrielsen *et al.*, 2012). Tpx is present solely as a homodimer in the reduce state. Conformational changes were observed between the reduced and oxidized state at the dimer interface, which might affect the binding ability of salicylidene acylhydrazide. Besides, WrbA is an NAD(P)H: quinone oxidoreductase that catalyzes two-electron reduction of quinone compounds and prevents ROS production in *E. coli* (Patridge and Ferry, 2006). However, these proteins are located in the cytosol. Early study has found that outer membrane of Gram-negative bacteria prevents the permeation of salicylidene acylhydrazide compounds (Macielag *et al*., 1998). Therefore, it is unclear whether this group of compounds is able to transport across bacterial membrane to interact with its cytoplasmic targets.

### 1.5 Iron uptake system in *E. amylovora*

Bacteria have evolved an aggressive strategy for acquisition of iron, an essential nutrient (Raymond *et al*., 2003). To survive in iron deficient environment, bacteria release small molecule siderophore, which has high affinity of binding free or chelated iron. When siderophore binds ferric ions, the complex contacts cell membrane-bound receptors and then move into bacterial cells. In Gram-negative bacteria, transportation of chelated iron across outer membrane involves the inner membrane complex TonB/ExbB/ExbD, in which the translocation is dependent on proton motive force and ATP hydrolysis (Moeck and Coulton, 1998). This process is regulated by the regulator protein Fur or Fur-like proteins.

*E. amylovora* is known to only synthesize the trihydroxamate siderophore, which belongs to a desferrioxamine (DFO) family (van der Zwet *et al*., 2011). The genes encoding the component proteins involving iron uptake and transportation have been identified in *E. amylovora* genome. These genes include DFO biosynthesis protein (*dfoJAC*), TonB protein (tonB) and its dependent receptors (*foxR, EAM_1087, EAM_1778, EAM_1726*), iron ABC transporters (*hmuSTUV, fhuCDB,* and *sitABCD*) (Smits *et al*., 2010). Early studies showed that siderophore DFO was required for *E. amylovora* virulence. Using *in vivo* expression technology (IVET), Zhao *et al.* (2005) identified a few iron-uptake genes of *E. amylovora* that were specifically upregulated when immature pear tissue was inoculated with bacterium. These included *ftn* (iron storage protein), *c3774* (ferric transport protein), *btuB* (iron transport), *exbB* and *hemT*. Similarly, Yang *et al*. (2004) observed induction of iron-acquisition genes of *Erwinia chrysanthemi* in response to spinach leaves, such as iron transporter genes *humSTU* and *yfeA*. Clearly, *Erwinia* species rely on iron acquisition system to adapt environment inside host plant. To test whether such a host-induced iron uptake is directly related to *E. amylovora* infection, Dellagi *et al.* (1998) generated mutant strains disrupting *dfoA* and *foxR*, respectively. *dfoA* is a DFO biosynthetic gene, while *foxR* encodes TonB-dependent receptor. It was found that the two mutants exhibited reduced symptoms as well as bacterial multiplication when infected apple blossoms were compared to the wild type. Collectively, these findings illustrated an important role of the iron uptake system in *E. amylovora* virulence.

### 1.6 Antibiotic-mediated activation of iron uptake system

Activation of iron uptake system has been directly related to ROS formation in antibiotic-induced cellular response. Dwyer *et al.* (2007) conducted a microarray analysis to determine bacterial adaption mechanism in response to antibiotics that cause DNA gyrase poisoning. Combined with *E. coli* microarray data under a variety of conditions, a set of genes that experienced statistically significant changes after antibiotic treatments were identified. Gyrase inhibition resulted in a significant upregulation of genes in DNA damage response (e.g. *UmuCD, DinDFGI*), oxidative stress response (e.g. *SodA, SoxS*) and iron uptake system (e.g. *ExbBD, TonB, IscARSU*). Gyrase poisoning is known to

cause superoxide production, leading to cycling disruption of Fe–S clusters between oxidized and reduced states. Destabilized Fe-S cluster results in the release of ferrous iron. The "free" ferrous iron then catalyzes Fenton reaction and generates destructive hydroxyl radicals, which contribute to cell death. Since superoxide and hydroxyl radical are important agents of reactive oxygen species (ROS), disruption of iron regulatory dynamics plays an important role in antibiotic-mediated cell death through ROS action.

Later reports suggest that breakdown of iron regulatory cycling is associated with a common mechanism of cell death shared by different classes of antibiotics. Despite that the majority of bactericide drugs have different macromolecule targets, production of hydroxyl radical via Fenton reaction is commonly observed in antibiotic treatments. This phenomenon is dependent on the presence of free iron, which results from destabilization of iron-sulfur clusters. In bacterial transcriptome, upregulation of iron acquisition system serves as an indicator of disruption of iron regulatory cycling after antibiotic exposure. It is now clear that antibiotics disruptsthe TCA cycle and cause a rapid NADH depletion, leading to superoxide formation. The superoxide then attack iron-sulfur clusters, resulting in a release of free iron, which are crucial catalytic agent of hydroxyl radical formation via Fenton chemistry (Kohanski *et al*., 2007; Davies *et al*., 2009).

Recent studies have provided abundant evidence to support the role of small molecule inhibitors in the interference of T3SS function of animal pathogenic bacterium (Nordfelthn *et al*., 2005; Dahlgren *et al*., 2007; Kauppi *et al*., 2007). In this context, small molecule inhibitors provide a new direction for development of new antimicrobial compounds that might disarm rather than kill bacteria. Since T3SS of *E. amylovora* shares a high level of identity with those T3SSs in other Gram-negative bacteria, it is believed that small molecules effective against animal and human pathogens may also work for *E. amylovora*. Furthermore, no such research has been conducted in plant pathogenic bacteria. This study is designed to screen for compounds that specifically target *E. amylovora* T3SS and disease development. This information could be useful for future drug discovery and potential management of fire blight. The main purpose of this project is to identify and characterize small molecules that inhibit T3SS of *E. amylovora.* The specific objectives of this project are:

- To screen small molecule compounds as virulence blockers against T3SS in *E. amylovora*
- To evaluate the effectiveness of small molecules in inhibiting T3SS gene expression and protein secretion

# CHAPTER 2

## SCREENING T3SS INHIBITORS OF ERWINIA AMYLOVORA


### Abstract

Type III secretion system (T3SS) is a potent virulence mechanism shared by a broad spectrum of Gram-negative bacteria that infect both plant and mammalian hosts. Plant pathogenic bacteria utilize T3SS to inject effector proteins into plant host cells, thus manipulating the host immune response. It is reasonable to believe that disabling the T3SS function may provide another way of controlling bacterial diseases. High-throughput screening of chemical libraries have identified small molecule inhibitors that attenuate T3SS of mammalian pathogens, but no study has been reported so far for plant pathogenic bacteria. In this study, three small molecules were identified to delay hypersensitive response (HR) development in tobacco. Using GFP as a reporter, six chemicals were identified to suppress T3SS gene expression of *E. amylovora* under *hrp* inducing conditions. Five of them belong to salicylidene acylhydrazides. Among them, chemical # 3 and # 9 were most potent in inhibiting T3SS gene expression in a dose-dependent manner without affecting bacterial growth. Furthermore, this group of chemicals also inhibited exopolysaccharide amylovoran production, another virulence factor in *E. amylovora*. Our results further demonstrated that chemical # 3 treatment resulted in growth reduction and symptom suppression on crab apple blossom.


### 2.1 Introduction

Fire blight, caused by Gram-negative bacterium *E. amylovora*, is a serious bacterial disease in Rosaceae family plants (Zhao *et al*., 2005). It mainly infects three economically important pome fruit trees, apple (*Malus sylvestris*), pear (*Pyrus communis* L.) and quince (*Cydonia oblonga* Mill). Fire blight was first reported in New York in 1780. In the U.S., financial loss due to fire blight contributes to production costs of about $100 million dollars annually. The characteristic symptoms of fire blight can be easily recognized, because of the appearance of blackening tissue as though they had been scorched by fire (Schroth *et al*., 1974). After blossom turns brownish or black following disease establishment, infection can progress rapidly along shoot and twig, resulting in

ooze drop or cobweb-like bacterial stand under wet conditions. Shoot blight also forms typical shepherd's crook symptom at wilting tips, in which blackening of leaf midrib is often present. Fruit blight is associated with prematurely red or green margin along the necrotic area. Finally, infection may spread downward into larger limbs or trunk, in which canker extension kills part of limb or the whole tree (van der Zwet *et al*., 2011).

Current fire blight management mainly relies on copper compounds and antibiotics application. Streptomycin, a compound belonging to aminoglycoside family, has been the most potent chemical agent in controlling fire blight in apple and pear orchards. In the past years, however, streptomycin-resistant *E. amylovora* strains became prevalent in many places including Washington, New York, Oregon, Missouri, Michigan and Canada (Russo *et al.*, 2008; Loper *et al*., 1991; Coyier and Covey, 1975; Shaffer and Goodman, 1985; Manulis *et al*., 1998; Norelli *et al*. 2003; McGhee and Sundin, 2011). Therefore, developing new antibacterial agents for prevention or treatment of fire blight becomes necessary.

*E. amylovora* is a short rod filamentous Gram-negative bacterium. Like many other Enterobacteriaceae, *E. amylovora* employs a type III secretion system (T3SS) to suppress plant immune response. In *E. amylovora*, T3SS is encoded by the *hrp* gene cluster, which is required to elicit hypersensitive response (HR) on non-host plant and cause disease on susceptible host plants. The *hrp* gene cluster is located on a 62 kb genomic DNA pathogenicity island 1 (PAI-1). When the environment condition is suitable for infection, the bacteria builds a functional T3SS device and injects effector proteins into host cells. Our previous study showed that Δ*T3SS* mutant strain failed to cause symptom or grow on immature pear fruits, indicating that T3SS is required for a successful infection of *E. amylovora* (Zhao *et al*., 2009).

T3SS has become a logical target for chemotherapeutic intervention, because its homologous macromolecular apparatus has been identified only in Gram-negative bacteria and is a key virulence determinant in many plant and mammalian pathogens (Baron, 2010). To identify compounds specifically blocking T3SS, many laboratories conducted chemical screening targeting known T3SS processes, including gene expression, effector secretion and translocation, and symptom development. These screens have led to the identification of several classes of T3SS-inhibiting compounds.

Kauppi *et al*. (2003) constructed a screening strain of *Y. pseudotuberculosis* that carries luciferase-encoding hybrid gene *luxAB* under the control of effector *yopE* promoter. From a 9,400-compound library, two salicylidene acylhydrazides were found to strongly suppress *yop* gene expression and protein secretion, as well as flagellum assembly and motility. Structural-function relationship assay suggested many compounds from this family shared similar biological activities against T3SS in *Yersinia* (Nordfelthn *et al*., 2005; Dahlgren *et al*., 2007; Kauppi *et al*., 2007). Later studies have shown that this family compounds broadly interfere T3SS function in different animal pathogenic bacteria, including *Salmonella typhimurium*, *E. coli* O157: H7, enteropathogenic *E. coli* (EPEC), *Shigella flexneri* and *Chlamydia* spp. (Tree *et al*. 2009; Hudson *et al*. 2007; Negrea *et al*. 2007; Muschiol *et al*. 2009; Veenendaal *et al*. 2009; Layton *et al*. 2010). Most recently, salicylidene acylhydrazide INP0341 was found to protect mice from vaginal infection of *C. trachomatis*, showing a potential role as vaginal microbicide (Slepenkin *et al*., 2011). Other classes of small molecule inhibitors of T3SS have also been reported (Izoré *et al*., 2011). 2-imino-5-arylidene thiazolidinone was known to block T3SS protein secretion of *S. typhimurium* and *Y.enterocolitica*, and also prevented HR when *P. syringae* were co-infiltrated intotobacco (Felise *et al*., 2008). Aurodox, a linear polyketide compound isolated from Streptomyces sp. K01-0509, was recently reported to inhibit protein secretion of *Citrobacter rodentium*. Oral administration of aurodox contributed to the survival of mice that had received a lethal dose of *C. rodentium*, which was consistent with intestine pathological features. However, it is unclear whether these T3SS inhibitors are effective against plant pathogenic bacteria.

In this study, two independent chemical screens were established and enabled identification of small molecules specifically targeting *E. amylovora* T3SS functions. Three chemicals (# 6, # 8, and # 11) were found to inhibit HR development on tobacco. Under a defined *hrp* inducing condition, six compounds (# 1-4, # 9 and # 12) were found to significantly suppress promoter activities of T3SS genes using GFP as a reporter. The effects of these compounds on EPS amylovoran production and *in vivo* infection assay were also characterized. Our analyses showed that the chemical screen was a powerful tool in identifying compounds that inhibit T3SS.

## 2.2 Materials and methods

### 2.2.1 Bacterial strains and growth condition

The bacterial strains and plasmids used in this study are summarized in Table 2.1. LB medium is used routinely for culturing *E. amylovora*. When necessary, the following antibiotics were added to the medium: 50 μg/mL kanamycin (Km), 100 μg/mL ampicillin (Ap) and 50 μg/mL rifampicin (Ri). Amylovoran production was determined by growing bacteria in MBMA medium (3 g $KH_2PO_4$, 7 g $K_2HPO_4$, 1 g $[NH_4]_2SO_4$, 2 ml glycerol, 0.5 g citric acid, 0.03 g $MgSO_4$) amended with 1% sorbitol (Zhao *et al*., 2009).

**Table 2.1 Bacterial strains, plasmids, and primers used in this study**

| strains or plasmids | Relevant characters[a] | Reference or source |
|---|---|---|
| ***E.amylovora* strains** | | |
| **Ea1189** | Wild type, isolated from apple | Burse *et al*. (2004) |
| **Ea273** | Wild type, isolated from apple | Bogdanover *et al*. (1998) |
| **Z623** | $Ri^R$ -resistant mutant of Ea273, $Ri^R$ | This study |
| **Z538** | dspE::Km; $Km^R$-insertional mutant of dspE of Ea1189, $Km^R$ | Zhao & Sundin (2008) |
| **Z133** | T3SS island::Km; $Km^R$ -insertional mutant of T3SS island of Ea1189, $Km^R$ | Zhao & Sundin (2008) |
| **Plasmids** | | |
| **pFPV25** | $Ap^R$, GFP-based promoter trap vector containing a promoterless gfpmut3a gene | Valdivia & Falkow (1997) |
| **pHrpN** | 735-bp EcoRI-BamHI DNA fragment containing promoter sequence of hrpN gene in pFPV25 | This study |
| **pHrpA** | 708-bp EcoRI-BamHI DNA fragment containing promoter sequence of hrpA gene in pFPV25 | This study |
| **pZW2** | 608-bp KpnI-XbaI DNA fragment containing promoter sequence of hrpL gene of Ea273 in pFPV25 | Wang *et al.* (2010) |
| **pZW3** | 570-bp SmaI DNA fragment containing promoter sequence of dspE gene of Ea273 in pFPV25 in forward orientation | Wang *et al.* (2010) |
| **Primers** | | |
| **hrpN1** | CCGGAATTCAAAGCGCTTCCTGTTTACTGC (*EcoR*I) | This study |
| **hrpN2** | CGCGGATCCGCTCCCAGCCCACTTGTAT (*BamH*I) | This study |
| **hrpA1** | CCGGAATTCGTGAAAACGTCAGGCAGCTA (*EcoR*I) | This study |
| **hrpA2** | CGCGGATCCCCTGTAATAATGCCGCTCAT (*BamH*I) | This study |
| **hrpL1** | CGGGGTACCTCCTCCATTGAGTCCTCCAG (*Kpn*I) | Wang *et al*. (2010) |
| **hrpL2** | CTAGTCTAGACCAGGTCATTTGCTCCAGAT (*Xba*I) | Wang *et al.* (2010) |
| **dspE1** | TCCCCCGGGCAGTGAGGGGGGGCAGACTTTTTTTTAACC (*Sma*I) | Wang *et al.* (2010) |
| **dspE2** | TCCCCCGGGTATCTTCGCCGCTGCCACCTTTCACCATTG (*Sma*I) | Wang *et al.* (2010) |

a. $Km^R$ =Kanamycin resistance, $Ap^R$ = ampicillin resistance, $Ri^R$ = rifampicin resistance; GFP = green fluorescent protein. Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primer.

### 2.2.2 HR assay

*E. amylovora* Ea1189 strain was grown overnight at 28 ℃. Cells were resuspended to $OD_{600}$ = 0.15 in sterile phosphate buffered-saline (PBS). Chemicals were

added to bacterial suspension to a final concentration of 100, 50, 10 μmol, and DMSO was added as negative control. The mixture was infiltrated into tobacco (*Nicotiana benthamiana*) leaves by needle-less syringe. Infiltrated plants were kept in a humid growth chamber, and HR was recorded at 24 or 48 h post infiltration.

### 2.2.3 Construction of promoter-GFP fusion for promoter activity assay

The promoter region of T3SS genes *hrpN* and *hrpA* was amplified by PCR. Primer pairs hrpN1-hrpN2 and hrpA1-hrpA2 with restriction sites were used to amplify 735 bp *hrpN* and 708 bp *hrpA* promoter sequences from *E. amylovora* WT strain, respectively. PCR products and the promoter trapping vector pFPV25 were both digested with *Eco*RI and *Bam*HI. The resulting fragments were gel-purified, ligated together, and cloned to the upstream of promoterless GFP gene. The final plasmids were designated as pHrpN for *hrpN* and pHrpA for *hrpA*, which were confirmed by restriction enzyme digestion and sequencing.

### 2.2.4 GFP reporter assay by flow cytometry

The BD FACSCanto flow cytometer was used to monitor the GFP intensity of strains containing the corresponding promoter-GFP construct (Zhao *et al.*, 2009). For *hrp* inducing condition assay, strains containing GFP-promoter fusion plasmids were grown in LB overnight and washed twice by PBS. The bacterial suspension was re-inoculated into *Hrp*-inducing minimal medium (HMM) (1g $[NH_4]_2SO_4$, 0.346 g $MgCl_2.6H_2O$, 0.099 g NaCl, 8.708 g $K_2HPO_4$, 6.804 g $KH_2PO_4$) or M9 minimal medium (12.8g $Na_2HPO_4.7H_2O$, 3.0 g $KH_2PO_4$, 0.5 g NaCl, 1g $NH_4Cl$, 0.24g $MgSO_4$, 0.011g $CaCl_2$). HMM and M9 were further supplemented with different carbon sources at different concentrations. Bacteria cultures were harvested by centrifugation at different time points, and resuspended in PBS for flow cytometry. For chemical screening, strains containing GFP-promoter fusion plasmids were washed by PBS and resuspended in HMM containing 20 mmol galactose. One ml cell suspension was seeded into 24-well plates at a density of $OD_{600}$ 0.2. Compounds were added into each well to yield a final concentration of 100 μmol, while DMSO was added as controls or water as no treatment. The GFP intensities were measured by flow cytometry after incubation at 18 ℃ for 18 h. For *in*

*vivo* gene expression assay, chemicals were added into a bacterial suspension to yield a final concentration of 100 μmol, while DMSO was added as controls or water as no treatment. The immature pear fruits were cut in half, and the resulting bacterial suspensions were directly added to the surface. After incubation at 26 ℃ for 10 h, bacterial cells were collected by either washing or centrifugation, washed twice and resuspended in PBS for flow cytometry assay. Flow cytometry was performed on a BD LSRII 10 parameter multilaser analyzers (BD Bioscience, San Jose, CA). For both cases, data were collected for a total of 100,000 events and statistically analyzed by gating using flow cytometry software FCS Express V3 (De Novo Software, LA, CA). A geometric mean was calculated for each sample. Each treatment was performed in triplicate and each experiment was repeated three times.

### 2.2.5 CPC assay for determining amylovoran concentration

The amylovoran concentration in supernatants of bacterial cultures was determined quantitatively by a turbidity assay with CPC, as described previously (Bellemann *et al.*, 1994; Hildebrand *et al*., 2006). Briefly, wild type *E. amylovora* Ea273 was grown overnight in LB broth and washed with PBS three times. After the final wash, the bacterial pellet was resuspended in 200 μl of PBS. A total of 100 μL of bacterial cell was inoculated into 10 ml of MBMA medium with 1% sorbitol. 3 ml resulting cell suspension was seeded into falcon tubes. Chemicals were added into bacterial suspension to a final concentration of 25 μmol, while DMSO was added as controls or water as no treatment. After incubation for 24 h at 28 °C with shaking, 50 μl of CPC at 50 mg/ml was added to 1 ml of supernatant following centrifugation. After 10 min of incubation at room temperature, amylovoran concentration was determined by measuring $OD_{600}$ turbidity. The final concentration of amylovoran production was normalized for a cell density of 1.0. For each strain tested, the experiment was repeated at least three times.

### 2.2.6 Crab apple flower assay

For *E. amylovora* WT and mutants, bacterial suspensions were grown overnight in LB broth, harvested by centrifugation, and resuspended in PBS at a density of $OD_{600}$

0.2. Bacteria were incubated with compound at 50 µmol or equivalent volume DMSO for 4 h at room temperature (RT) in the dark. One day old fully opened crab apple (*Malus mandshurica*) flower were detached, and immediately transferred to microcentrifuge tube containing 2 ml of 10% sucrose. Two µl bacterial suspensions were evenly delivered to stigmas per flower by pipette under microscope. The inoculated flowers were incubated in a growth chamber at 24 ℃ with 90% relative humidity in a sealed container. Symptoms were recorded at 6 day post inoculation. For bacterial population assay, flowers were ground at 0, 2, 4, and 6 days post inoculation. Population was measured by dilution plating and colony-forming unit (CFU) was calculated.

## 2.3 Results

### 2.3.1 Small molecules that delay HR development in tobacco

In *E. amylovora*, HR inducing activity requires a functional T3SS, especially the translocation of elicitor HrpN (Wei *et al*., 1992). To identify compounds that disrupt T3SS activity in *E. amylovora*, a total of 16 small molecules were screened on tobacco plants. At concentration of 50 µmol or above, three chemicals (# 6, 8, and 11) inhibited HR development post 24 h infiltration (Fig.2.1). Normal tissue collapse was observed for all other chemicals at all concentrations as that of control DMSO.

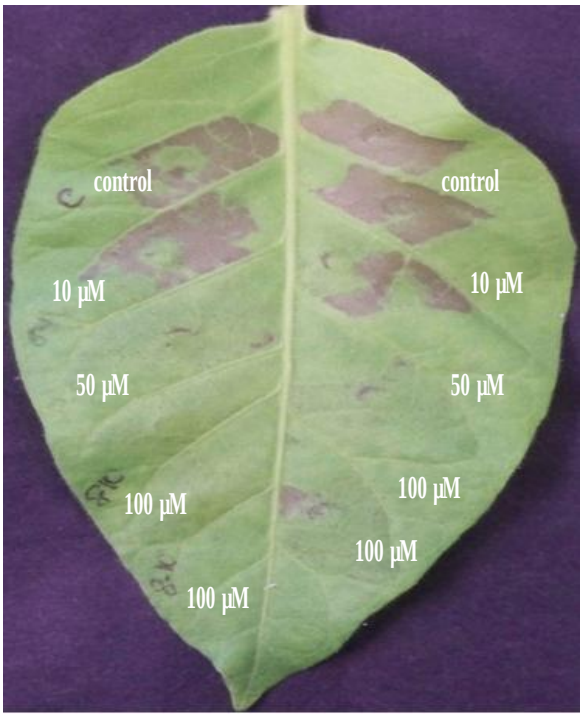


**Fig. 2.1 Small molecules delayed hypersensitive response (HR)**. Tobacco leaf was infiltrated with *E. amylovora* Ea1189 ($OD_{600}$ = 0.15) in the presence of compound # 8 (left panel) and # 11 (right panel) at the concentrations indicated. The same volume of DMSO was added as control. The image was taken at 24 h post infiltration.

### 2.3.2 Defining *hrp* inducing condition

Different carbon sources were used to induce *hrp* genes expression in *E. amylovora*. These carbon sources included galactose, glucose, mannitol and fructose at 10 and 20 mmol. In the M9 minimal medium, the GFP intensity of *dspE* promoter was 7.22 when bacteria were grown in the presence of 10 mmol galactose. At the same concentration, GFP intensity for *dspE* promoter was 4.51, 3.49 and 4.35 in glucose, mannitol and fructose, respectively. On the other hand, the GFP intensity for *dspE* promoter was 9.55, 5.75, 5.22 and 6.56 when grown in HMM containing 10 mmol galactose, glucose, mannitol and fructose, respectively. These results demonstrated that HMM containing galactose had a stronger inducing effect on *dspE* promoter activity than any other carbon sources tested in both media (Fig. 2.2(A)).

A significant increase in promoter activity was observed at higher concentration of carbon sources. The GFP intensity for *hrpA* promoter was 24.63 and 25.19 when grown in M9 minimal medium containing 10 and 20 mmol galactose, respectively; whereas its activity in HMM containing 10 and 20 mmol galactose was 32.51 and 34.81, respectively. Similar inducing effects were obtained for promoter activities of *hrpN*, *dspE* and *hrpL* genes, which reached the highest levels when strains were grown in HMM containing20 mmol galactose at 18 ℃ for 18 h, and the GFP intensity for *hrpN*, *dspE* and *hrpL* promoters was 11.63, 17.87 and 10.06, respectively (Fig. 2.2(B)). Thus, HMM containing 20 mmol galactose was chosen as a working medium.

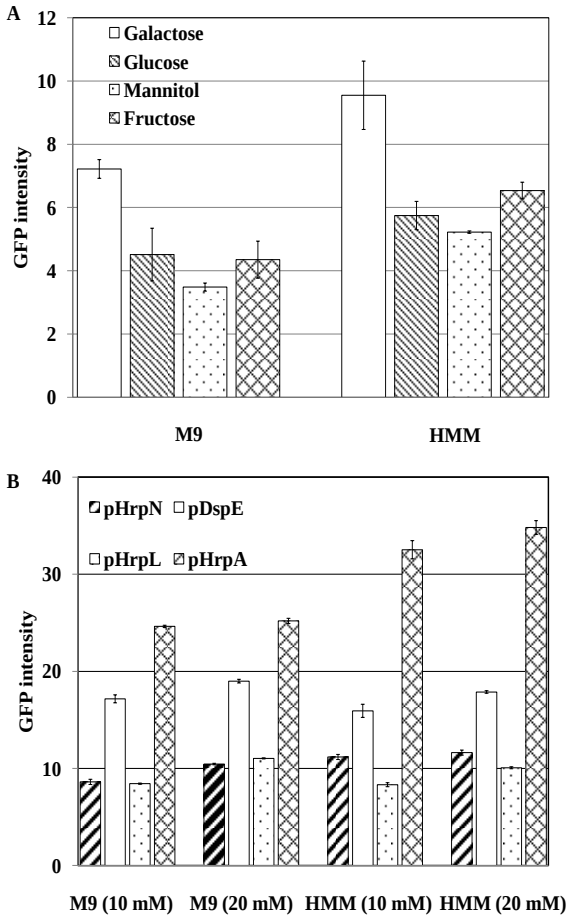


**Fig. 2.2 Promoter activities of T3SS genes of *E. amylovora in vitro*. A,** Promoter activity of *dspE* in *E. amylovora* grown in HMM or M9 medium containing different carbon sources. **B,** Promoter activities of *hrpN*, *dspE*, *hrpL*, *hrpA* in *E. amylovora* at different concentration of galactose.

Next, we determined the time course of the promoter activities for four genes in the working medium. After 2 h incubation, the GFP intensity was 1.72, 1.89, 1.60 and 2.40 for *hrpN*, *dspE*, *hrpL* and *hrpA* promoter, respectively. The promoter activities for these genes increased after 8 h incubation and reached highest level at 18 h, and then remained steady at 24 h. The GFP intensity at 18 h was 10.76, 15.55, 8.56 and 34.31, respectively for *hrpN*, *dspE*, *hrpL* and *hrpA* promoter, and was 11.63, 17.87, 10.06 and 34.81, respectively, at 24 h. Thus, 18 h was chosen to measure the GFP activity in the following screening assays.

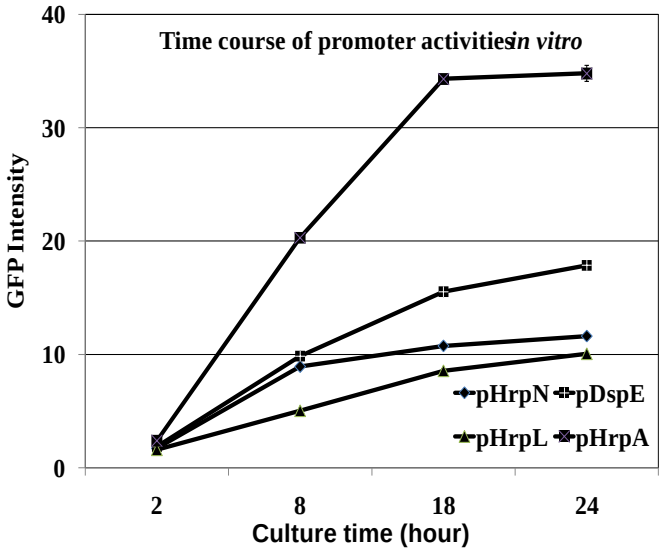


**Fig. 2.3 Time course of promoter activities of *hrpN, dspE, hrpL,* and *hrpA*.** Strains were grown in HMM containing 20 mM galactose.

### 2.3.3 Screening small molecules that inhibit T3SS gene expression *in vitro*

A total of 16 small molecules were tested for inhibition of promoter activities of *hrpN*, *dspE*, *hrpA* and *hrpL* genes under *hrp* inducing conditions as described above. The GFP intensity for *hrpN*, *dspE*, *hrpA* and *hrpL* promoters were 11.76, 16.87, 42.17, 10.66, respectively, in no-treatment samples, and were 12.33, 19.00, 43.68 and 9.66, respectively, when bacteria were grown in the presence of DMSO. Therefore, addition of equivalent volume of DMSO had no effect on promoter activities of those genes (Fig 2.4).

Significant inhibition of *hrpN*, *dspE*, *hrpA* and *hrpL* promoter activities was observed with chemicals # 1-4 and # 9, a group of salicylidene acylhydrazide. Among them, chemicals # 3 and # 9 were the most effective, followed by # 1, # 2 and # 4. The GFP intensity for *hrpN* promoter after chemical # 3, # 9, # 1, # 2 and # 4 treatment was 3.27, 3.83, 3.86, 5.26 and 8.85, respectively. The GFP intensity for *dspE* promoter after chemical # 3, # 9, # 1, # 2 and # 4 treatment was 3.49, 4.86, 4.57, 5.41 and 11.99, respectively. The GFP intensity for *hrpL* after chemical # 3, # 9, # 1, # 2 and # 4 treatment was 2.13, 2.10, 2.35, 2.68 and 4.74, respectively. Similarly, the GFP intensity for *hrpA* promoter after chemical # 3, # 9, # 1, # 2 and # 4treatment was 8.69, 8.23, 8.76, 13.86 and 25.99, respectively. Another compound # 12, a bromobenzoate, also inhibited promoter activities of *hrpN*, *dspE*, *hrpA* and *hrpL*. The GFP intensity was 6.39, 7.18, 12.13, and 3.44, for *hrpN*, *dspE*, *hrpA* and *hrpL*, respectively. Other compounds, however, had no or little effect on promoter activities of the four genes.

At a range of concentrations (10 to 100 μmol), analysis of effectiveness showed that chemicals # 3 and # 9 showed a dose-dependent inhibition of *hrpN*, *dspE*, *hrpA* promoter activities (Fig. 2.5). For example, after treatment of chemical # 3 at 10, 25, 50 and 100 μM, the GFP intensity for *hrpN* promoter was 5.50, 4.38, 4.07 and 3.27, respectively, while of the GFP intensity for DMSO control was 11.52. Under the same culture condition, the GFP intensity for *hrpN* promoter was 6.06, 5.89, 5.04 and 3.83, respectively after treatment of chemical # 9 at 10, 25, 50 and 100 μM. However, only minor change in the *hrpL* promoter activities was observed when bacteria were grown in the presence of different concentrations of chemicals # 3 and # 9. The GFP intensity for *hrpL* promoter was 2.51, 2.39, 2.41 and 2.13, respectively, after treatment of chemical # 3 at 10, 25, 50 and 100 μM; and was 2.52, 2.34, 2.17 and 2.10, respectively after treatment of chemical # 9 at 10, 25, 50 and 100 μM.

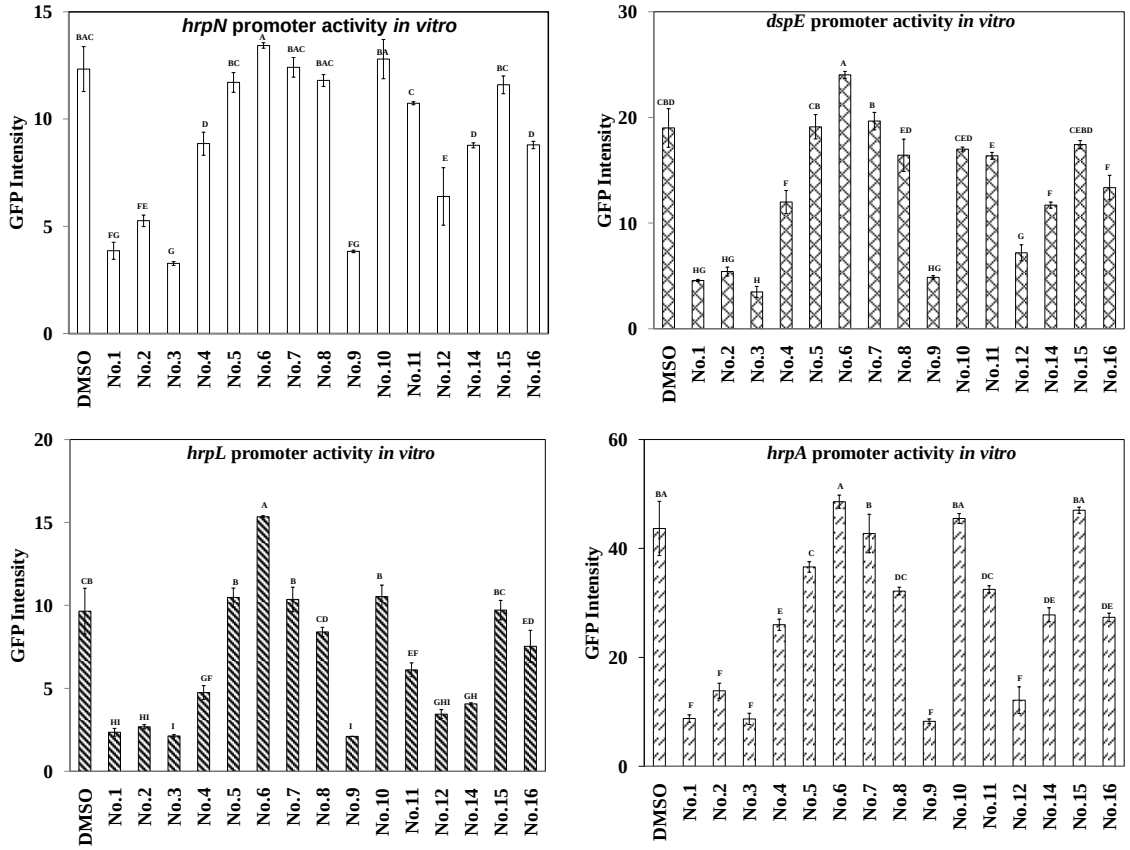


**Fig. 2.4 Effect of small molecules on promoter activities of *hrpN*, *dspE*, *hrpL*, *hrpA*.** Strains were grown in HMM in the presence of 100 μmol of chemicals. The equivalent volume of DMSO added as control or water as no treatment. NT: no treatment. Error bars indicate standard deviation. One way ANOVA and the Turkey's W test (P = 0.05) was carried out to determine difference in means using SAS program.

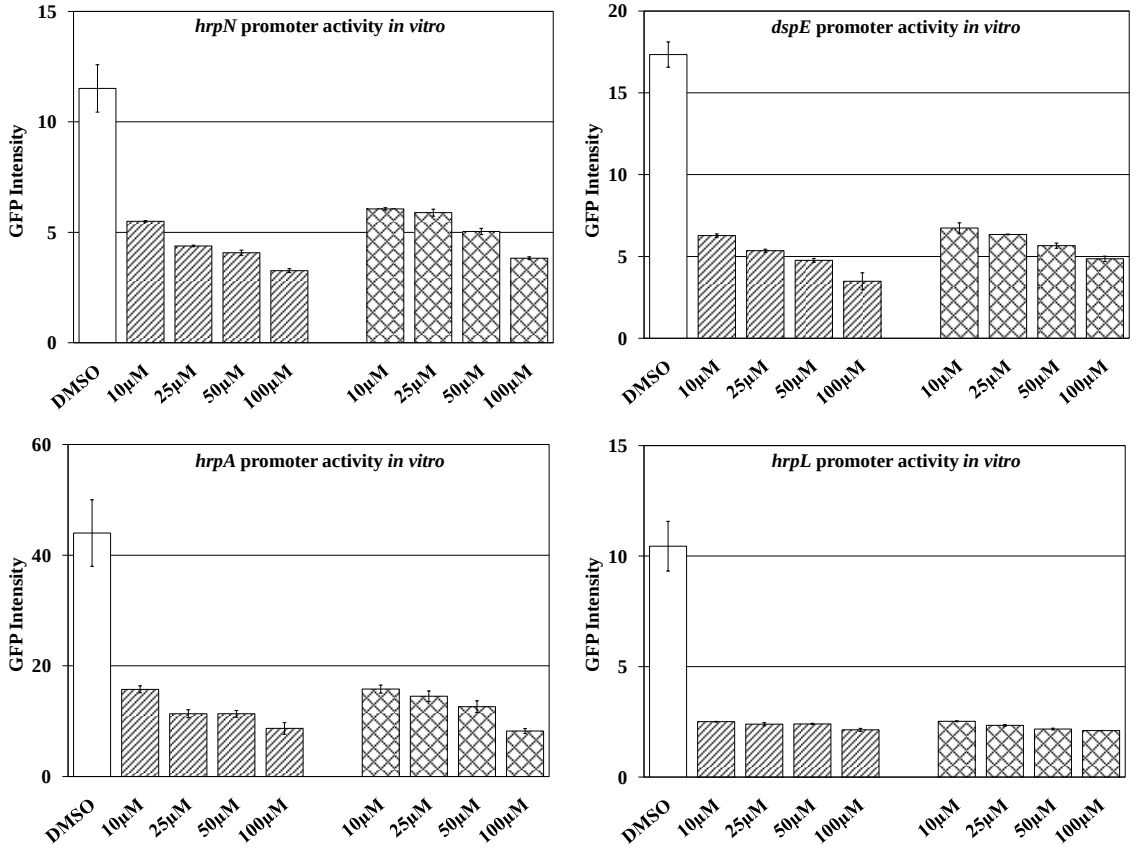


**Fig. 2.5 Effect of small molecules on promoter activities of T3SS genes at different concentrations.** Strains were grown in HMM 18 ℃ for 18 h. DMSO, empty bars; # 3 strip bars; and # 9 diamond bars.

### 2.3.4 Compounds # 3 and # 9 did not inhibit *E. amylovora* growth *in vitro*

To eliminate the possibility that compounds # 3 and # 9 caused a growth defect of *E. amylovora*, colony-forming units (CFUs) of bacterial cultures were monitored in the presence of compounds under the *hrp* inducing condition. At the concentration of 25 μmol, the two compounds caused negligible alteration in *E. amylovora* growth (Fig.2.6).

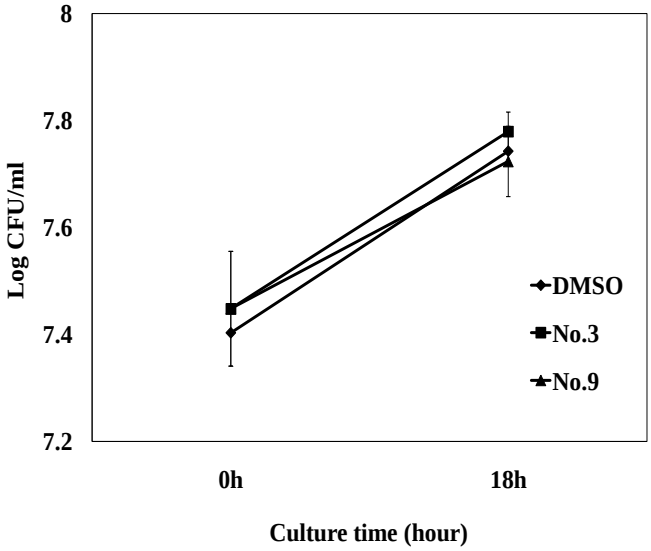


**Fig. 2.6 Effect of small molecules on bacterial growth.** Growth of *E. amylovora* was determined in HMM at 18 ℃ for 18 h**.**

### 2.3.5 Small molecules inhibited T3SS gene expression *in vivo*

To investigate whether small molecules are effective under *in vivo* conditions, strains containing GFP-promoter fusion plasmids were incubated with selected compounds or DMSO at a final concentration of 100 μmol in the dark for 1 h. The mixture was inoculated onto immature pear fruits and GFP intensity was measured. The GFP intensity for *hrpN* promoter *in vivo* was 15.85 and 24.37 for DMSO control or water treatment, respectively. In the presence of chemicals # 1, # 2, # 3, # 4 and # 9, the GFP intensity for *hrpN* promoter was 18.52, 15.20, 13.30, 17.92 and 17.63, respectively (Fig. 2.7). On the other hand, the GFP intensity for *hrpA* promoter for water control, DMSO, # 1, # 2, # 3, # 4 and # 9 treament was 52.74, 51.78, 26.31, 27.22, 26.22, 21.38 and 61.57, respectively. These results suggested, among salicylidene acylhydrazides, chemicals # 1-4 suppressed *hrpA* promoter activity but had no effect on *hrpN* promoter activity on immature pear fruit.

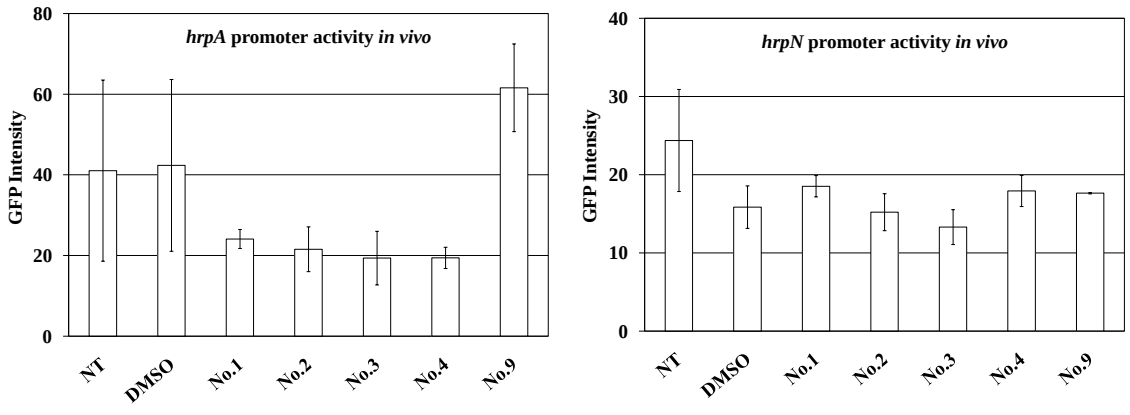


**Fig. 2.7 Effect of small molecule on promoter activities of *hrpA* and *hrpN* gene *in vivo*.** Small molecules affected promoter activities of *hrpL* and *hrpA* on immature pear fruits in the presence of 100 μmol of chemicals. The equivalent volume of DMSO added as control or water as no treatment. NT: no treatment.

### 2.3.6 Small molecules inhibited amylovoran production *in vivo*

To test whether small molecules could affect production of amylovoran, a cetylpyrimidinium chloride (CPC) assay was performed as described previously (Wang *et al*., 2010). At 25 μmol, amylovoran production for water control, DMSO, # 1, # 2, # 3, # 4 and # 9 treatment was 1.57, 1.40, 1.09, 1.09, 0.99, 1.19 and 1.32, respectively. Thus, chemical # 3 was the most potent inhibitor of amylovoran production.

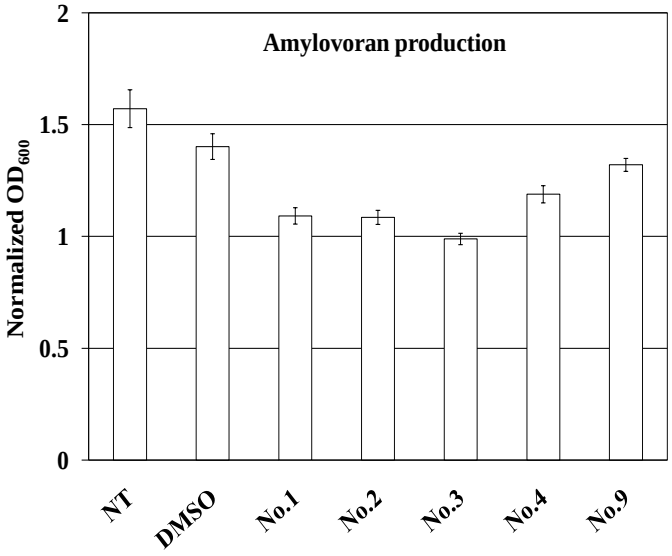


**Fig. 2.8 Effect of small molecules on amylovoran production.** *E. amylovora* stain Ea273 was grown in MBMA media with 1% sorbitol for 24 h at 28 ℃ with shaking. Chemical compounds, equal volume of DMSO or water were added to the cell culture at a final concentration of 25 μmol.

### 2.3.7 Chemical # 3 suppressed *E. amylovora* infection on crab apple flowers

To determine the effect of small molecules on symptom development and bacterial growth, a crab apple flower assay was carried out as described previously (Pusey, 1997). Six days post inoculation, there was a significant difference of symptom development in pistil when chemical # 3 coinoculated flowers were compared with those with DMSO alone (Fig. 2.9). The pistil of flower infected with *dspE* mutant remained healthy and showed the least necrosis near the point of inoculation. The compound # 3 coinoculated crab apple flowers exhibited a similar pattern in that only about one third of the pistils were slightly blackening. In contrast, bacterial-induced necrosis along the whole pistils was observed in the flowers co-inoculated with DMSO-treated or bacteria alone.


**Fig. 2.9 Virulence assay on crab apple (*Malus mandshurica*) flowers.** Symptoms of crab apple flower at 6$^{th}$ day post inoculation with *E. amylovora* stain Ea273, *dspE* mutant, Ea273 treated with DMSO or chemical # 3.

To investigate the effect of compound # 3 on *E. amylovora* multiplication *in vivo*, bacterial growth was also determined. Two days after inoculation, bacterial population was comparable between DMSO- and chemical # 3- treated flowers. After six days, treatment of chemical # 3 led to about two-fold decrease in bacterial population compared to that from flowers co-inoculated with DMSO alone (Fig. 2.10).

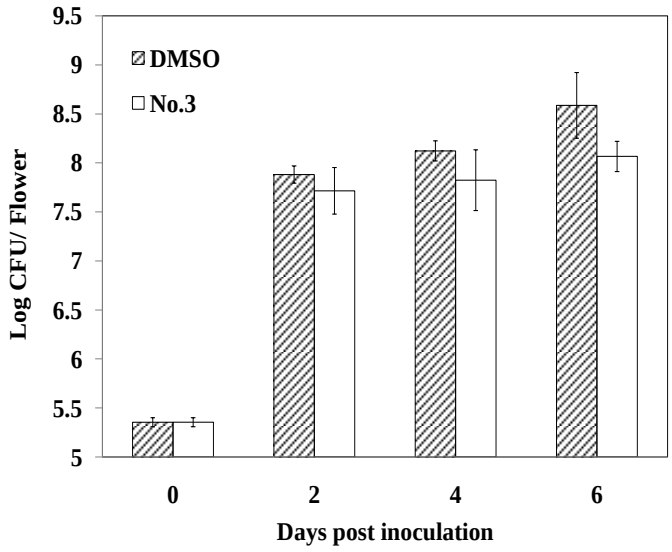


**Fig. 2.10 Effect of small molecule on bacterial growth on stigmas.** Flowers were ground at 0, 2, 4, and 6 days post inoculation. Population was measured by dilution plating and colony-forming unit (CFU) was calculated.

## 2.4 Discussion

High-throughput screen is a powerful tool in identifying small molecules that inhibit T3SS functions from synthetic or natural chemical library. Some of these compounds have been known to broadly impair T3SS function in a variety of Gram-negative bacteria. In this study, 16 small molecules were collected that are known as T3SS inhibitor in previous studies, and two independent chemical screenings were used to identify compounds that disrupt T3SS of *E. amylovora*.

Three compounds (# 6, # 8 and # 12) were identified as inhibitors of HR development on tobacco. Since Harpin protein HrpN is crucial in inducing plant cell death, it is possible that delayed HR might be due to disruption of secretion or translocation of HrpN by these three compounds. Felise *et al*. (2008) demonstrated that chemical # 6 (also known as compound 1) prevented HR when *P. syringae* were infiltrated into tobacco. They proposed that this compound widely interfered with T3SS function in many Gram-negative bacteria by disrupting needle complex formation or assembly. Chemical # 8 was also reported to effectively inhibit effector secretion in *Y.*

*pestis* and *EPEC*. Chemical # 12 was identified as a new T3SS inhibitor in this study. Future functional analysis is needed to test whether these three compounds affect HrpN secretion or needle complex assembly.

Six compounds were identified to specifically suppress transcription of T3SS genes under *hrp* inducing condition. Chemicals # 1-4 and # 9 belong to a family of salicylidene acylhydrazides. Chemicals # 3 and # 9 were the most effective, causing a dose-dependent inhibitory effect on T3SS gene expression without inhibiting bacterial growth. Interestingly, chemical # 3 was known to strongly inhibit expression of all T3SS genes and effector secretion in *E. coli* O157: H7, while chemical # 9 has been discovered as new T3SS inhibitor for the first time in this study. This result confirmed early studies that salicylidene acylhydrazides broadly inhibit T3SS gene transcription in *Y. pseudotuberculosis*, *S.typhimurium*, *E. coli* O157: H7, EPEC, *S. flexneri* and *Chlamydia* spp. Our results indicated that these chemicals also suppress T3SS gene expression in plant pathogenic bacteria, suggesting that a common mechanism is shared by salicylidene acylhydrazides in disrupting T3SS among different bacteria. Amylovoran is another virulence factor of *E. amylovora*, which is required for full pathogenesis of *E. amylovora*. Our results revealed that salicylidene acylhydrazides (# 1-4 and # 9) inhibited amylovoran production. Thus, these findings demonstrated, for the first time, that this family of chemicals is able to target different bacterial virulence factors. It is possible that this group of chemicals commonly act upstream of signal transduction that regulates T3SS function and amylovoran biosynthesis. Specifically, it has been reported that salicylanilide class compounds inhibit two component signal transduction systems in bacteria (Macielag *et al*. 1998). It has also been previously demonstrated that the RcsBCD phosphorelay system plays an essential role in controlling amylovoran production and partially influenced the expression of T3SS genes (Wang *et al*., 2012). Therefore, it is possible that salicylidene acylhydrazides may affect phosphorylation of the RcsBCD system.

Stigma inoculation allowed us to mimic chemical application to flowers in orchard and to evaluate the capability of the compounds in protecting blossoms from bacterial infection. Our results showed that chemical # 3 was able to protect crab apple flowers from *E. amylovora* infection. Among chemical # 3 treated flowers, necrosis was

limited near the inoculation points and bacterial growth was slightly inhibited than that of DMSO control. It is possible that the decreased infection on crab apple blossom is due to a decrease in effector protein secretion and amylovoran production after chemical # 3 treatment.

In conclusion, several T3SS inhibitors of *E. amylovora* were identified from two independent chemical screens. These chemicals exhibited variable abilities in affecting T3SS of *E. amylovora*: three compounds (# 6, # 8 and # 12) inhibited HR development on non-host plant, while six compounds (# 1-4, # 9 and # 12) inhibited T3SS gene expression *in vitro*. Our results also revealed that this group compounds have the ability to interfere with amylovoran production. Most strikingly, one compound from this group slightly protected crab apple blossom from *E. amylovora* infection. These findings showed that small molecule inhibitors could be explored for fire blight control. Further investigation such as transcriptome analysis and protein profiling assay are required to better understand the mode of action of these small molecules in virulence regulation of *E. amylovora*.

# CHAPTER 3

# GLOBAL TRANSCRIPTIONAL PROFILING INDUCED BY SMALL MOLECULE INHIBITORS


## Abstract

A group of salicylidene acylhydrazide compounds have been identified capable of inhibiting promoter activities of T3SS genes under *hrp*-inducing condition. These compounds also exhibited activity in inhibiting amylovoran production *in vitro*. To better understand the mode of action of this class of compounds, we carried out a microarray analysis of *E. amylovora* treated with chemicals # 3 and # 9. A total of 534 and 183 genes were identified to be significantly differentially regulated by chemicals # 3 and # 9 treatment, respectively. The majority of genes in *E. amylovora* T3SS cluster including *hrpL* as well as effectors including *avrRpt2* and *hopC1* were down-regulated more than two folds by both chemicals # 3 and # 9. Chemical # 3 also suppressed the transcription of all amylovoran biosynthesis genes. Interestingly, the most significant upregulated genes were those involving iron acquisition and utilization. In addition, other compounds of this group (chemicals # 1, # 2, # 4) also exhibited a similar effect on gene expression, i. e suppressing T3SS and *ams* gene expression, while promoting iron uptake gene expression. Our results suggested that a common inhibition mechanism may be shared by these compounds.


## 3.1 Introduction

*E. amylovora* is a gram-negative bacterium that causes fire blight on pome fruit trees, especially pear, apple and quince. The exopolysaccharide (EPS) amylovoran and type III secretion system (T3SS) are two main virulence factors. Amylovoran is a complex acidic heterpolysaccharide, consisting of galactose, glucose and pyruvate residues (Nimtz *et al*., 1996). *E. amylovora* contains 12 *ams* genes that are responsible for amylovoran biosynthesis, which is strictly regulated by the Rcs phosphorelay system (Wang *et al*., 2012; Zhao *et al*., 2009). T3SS is a structurally and functionally conserved macromolecular machine in many gram-negative bacteria, including animal and plant pathogens. Pathogens employ this protein secretion pathway to inject effector proteins

into eukaryotic host cells, thus manipulating host immune response. In *E. amylovora*, T3SS is encoded by the *hrp* gene cluster, which is required to elicit hypersensitive response (HR) on non-host plants and cause disease on susceptible host plants. Mutations of amylovoran biosynthesis or T3SS gene cluster rendered the bacterium nonpathogenic on host plants, suggesting that the two virulence factors are key determinants of *E. amylovora* infection (Zhao *et al*., 2005).

T3SS has become a logical target for chemotherapeutic intervention. A number of molecules were identified as T3SS inhibitors against many animal pathogenic bacteria. To identify compounds specifically blocking T3SS, many laboratories conducted chemical screening targeting known T3SS processes, including gene expression, effector secretion and translocation, and symptom development. These screens have led to the identification of several classes of T3SS-inhibiting compounds. Notably, a group of salicylidene acylhydrazides were reported to broadly inhibitT3SSfunction in *Y. pseudotuberculosis, S.typhimurium, E. coli* O157: H7, EPEC, *S. flexneri* and *Chlamydia* spp. However, the precise molecular mechanism of inhibition by salicylidene acylhydrazides remains unknown. For example, this group of compounds was apparently effective in suppressing T3SS gene transcription and effector secretion of *Y. pseudotuberculosis* (Kauppi *et al*., 2003; Nordfelthn *et al*., 2005; Dahlgren *et al*., 2007). Moreover, this group of compounds was known to block the translocation of effector IncG when the intracellular pathogen *C. trachomatis* infected host cell, causing a reduced number of bacterial inclusion body (Muschiol *et al*. 2006). After the treatment of salicylidene acylhydrazides, *S. flexneri* cells exhibited a high proportion of incomplete needle complex, indicating that this compound group might suppress needle complex formation or assembly. There is a need, therefore, to identify molecular target of salicylidene acylhydrazides in different bacteria and to decipher the molecular mechanism of inhibition.

In an effort to further understand the mode of action, researchers used microarray to determine global transcriptional responses following salicylidene acylhydrazides treatments. Microarray analysis showed that salicylidene acylhydrazides suppressed the expression of T3SS-1 genes of *S. typhimurium* (Layton *et al.*, 2010). About one quarter of the genes significantly changed after chemical treatment were involved in iron

acquisition and utilization. It was also found that this group of compounds suppressed expression level of all T3SS genes that were located on the locus of enterocyte effacement (LEE) pathogenicity island of *E. coli* O157: H7, as well as that of non-LEE effectors (Tree *et al*., 2009). Interestingly, addition of exogenous iron (ferrous or ferric form) had the ability to prevent the inhibitory effects on T3SS in both bacteria. Thus, it was proposed that this group compounds might cause iron depletion, resulting in inhibition of T3SS gene expression and protein secretion.

In this study, the effects of two structurally related salicylidene acylhydrazides on global gene expression in *E. amylovora* were characterized. Our analysis showed that transcriptional suppression of T3SS was accompanied by activation of genes encoding iron uptake and regulation. In addition, all amylovoran biosynthetic genes were down-regulated by chemical # 3, indicating that salicylidene acylhydrazides inhibited amylovoran production. Moreover, other compounds of this group (# 1, # 2, # 4) triggered a similar transcriptional pattern, indicating a common inhibition mechanism may be shared by this group compounds.

## 3.2 Materials and methods

### 3.2.1 RNA isolation

Wild type Ea273 was grown overnight in LB broth at 28 ℃. Cells were re-inoculated in HMM after washing twice by PBS. Cells were seeded into 12-well plates at a density of $OD_{600}$ = 0.3 cells/well in a volume of 2 ml. Compounds were added into each well at a final concentration of 50 μmol, and equal volume of DMSO was included as control. The plates were incubated at 18 ℃ for 18 h with shaking. 1.5 ml cultures were stabilized using 3 ml of RNA protect reagent (Qiagen, Hilden, Germany). The RNA was extracted using an RNeasy Mini Kit (QIAGEN). All RNA was quantified using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). And RNA quality was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, U.S.A.).

### 3.2.2 Microarray hybridization and data analysis

Microarray experiment and data analysis were carried out essentially as described previously (McNally *et al.*, 2011; Wang *et al*., 2012). In brief, the 8 × 15 k gene expression microarray of *E. amylovora* was designed at the James Hutton Institute and synthesized by Agilent Technologies. The detailed description of the oligonucleotide microarray is available at ArrayExpress website (accessions: microarray A-MEXP-2000).

Synthesis and labeling of cDNA was performed using a total of 10 μg RNA and FairPlay III microarray labeling kit (Stratagene, La Jolla, CA, U.S.A.), according to the manufacturer's instructions. After the labeling, the concentration of cDNA was determined by NanoDrop ND-100 spectrophotometer. The labeled cDNA (600 ng) was then hybridized to the slide for 17 h at 65 ℃ in an Agilent rotating oven (10 rpm) in the presence of a 2 × hybridization buffer (Agilent Technologies). After successive washing by gene expression wash buffers(Agilent Technologies), the hybridized slide was scanned using an Axon 4000B array scanner (Molecular Devices, Sunnyvale, CA, U.S.A.). Microarray images were processed by the GenePix Pro 6.0 image analysis software (vs. 6.0.1.26). Raw data were processed through logarithmical transformation and normalization by using R software. Statistical analysis using a multiple testing procedure was conducted to compare the statistical significance. Only the genes that showed at least two fold changes in gene expression ($\geq 2.0$ or $\leq 0.5$) were considered significant.

### 3.2.3 qRT-PCR

To validate the microarray data, gene expression level of *hrpA*, *dspE*, *hrpL*, *hrpN*, *avrRpt2*, *amsG*, *amsD* and *glgB*, *foxR*, and *EAM_3350* were determined by qRT-PCR. For each sample, synthesis of cDNA was performed with 1 μg of total RNA and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). Primers were designed using Primer3 software with high specificity and were listed in Table 3.1. qRT-PCR was conducted in the ABI 7300 System (Applied Biosystems, Foster City, CA, U.S.A.) using Fast SYBR Green PCR master mix (Applied Biosystems). All reactions were run on 96-well optical reaction plates. The thermal cycling conditions included a step of 2 min at 50 ℃ and 10 min at 95 ℃ followed by 40 cycles of 95 ℃ for 15 s and

72 °C for 30 s. The relative quantification (ΔΔCt) method was used to determine expression level of selected genes and16SrRNA (*rrsA*) gene was used as an endogenous control. A relative quantification (RQ) value for each gene was calculated and then normalized to those of DMSO samples. In order to determine if other salicylidene acylhydrazides (chemicals # 1, # 2 and # 4) exhibited similar effect on gene expression, RNA samples were isolated when wild type Ea273 was grown in the presence or absence of those chemicals.

**Table 3.1 Primers for qRT-PCR used in this study**

| Primers | Sequence (5'—3') |
|---|---|
| amsD1 | GATGCGTCTGTTCAAGCTGT |
| amsD2 | TCGCAACAAATCAGTCTGGA |
| amsG1 | CAAAGAGGTGCTGGAAGAGG |
| amsG2 | GTTCCATAGTTGCGGCAGTT |
| avrRpt2_1 | AAACCTTGCCGAAGTGAGTT |
| avrRpt2_2 | TTGCCAACCAAACATAATGG |
| dspE1 | TCCAGCGAGGGCATAATACT |
| dspE2 | ACAACCGTACCCTGCAAAAC |
| EAM_359_1 | GCACGATCTGGTGTTTATGC |
| EAM_359_2 | AATTTCTTCGCCGATGTCTT |
| EAM_3350_1 | ATCTCAGCCATTACCGCTTT |
| EAM_3350_2 | GCATACTGTTTCACCGCATT |
| foxR1 | CTTCGTGCCTATCTGCAAAA |
| foxR2 | AAACCGGTGCTCAGTTTTCT |
| glgB1 | GGGTTCAATTCTCGACCGTA |
| glgB2 | GGTGTCGTGGTTCCACTCTT |
| hmuS1 | TACAGCAGCAGGGTAACGAG |
| hmuS2 | TGTTCAGCCAGTTGTCCTTC |
| hrpA1 | GAGTCCATTTTGCCATCCAG |
| hrpA2 | TGGCAGGCAGTTCACTTACA |
| hrpN1 | GCTTTTGCCCATGATTTGTC |
| hrpN2 | CAACCCGTTCTTTCGTCAAT |
| hrpL1 | TTAAGGCAATGCCAAACACC |
| hrpL2 | GACGCGTGCATCATTTTATT |
| 16S3 | CCTCCAAGTCGACATCGTTT |
| 16S4 | TGTAGCGGTGAAATGCGTAG |

## 3.3 Results

### 3.3.1 Genes regulated by chemicals # 3 and # 9

A total of 534 and 183 genes were significantly differentially expressed in the chemicals # 3 and # 9 treatment, respectively. For the chemical # 3 treatment, 254 genes were upregulated and 280 genes were downregulated; whereas for the chemical # 9 treatment, 114 genes were upregulated and 119 genes were downregulated. Among them, 77 genes were commonly identified as being downregulated by both chemicals # 3 and # 9 treatments (Fig. 3.1). Further analysis revealed that majority of T3SS genes showed lower levels of expression after chemical exposure compared with DMSO treatment (Table 3.2). These genes include the regulatory gene (*hrpL*), the effector genes (*avrRpt2* and *hopC1*) and the secretion apparatus genes (*hsvB, hsvA, hrpD, hsvC, hrpI, hrpJ, hrpE, hrcN, hrcC, EAM_2876, hrpF, hrpV, hrcS, hrcJ, hrcQ, hrcU, hrpK, hrpO*) (Table 3.2).In addition, expression level of T3SS genes in chemical # 3 treatment was much lower than that of # 9 treatment, indicating that chemical # 3 was a more potent inhibitior of T3SS. For example, sigma factor encoding gene *hrpL* was downregulated by 0.16- and 0.28- by chemicals # 3 and # 9, respectively. Furthermore, chemical # 3 suppressed expression of all amylovoran biosynthesis genes (*amsL, amsK, amsJ, amsF, amsE, amsD, amsC, amsB, amsA, amsI, amsH, amsG*) (Table 3.3). All *ams* genes also exhibited reduced expression after # 9 exposure compared to DMSO treatment, but such changes were not statistically significant. These results were consistent with our previous findings that chemical # 3 was more effective in reducing amylovoran production than chemical # 9 did.

In addition, a total of 97 genes were commonly upregulated by chemicals # 3 and # 9.These included glycogen metabolism genes (*glgA, glgC, glgX, glgB*) and a group of genes involving iron acquisition, such as those encoding siderophore desferrioxamine E (*dfoJ, dfoA, dfoC*), TonB and its depedent receptor (*tonB, foxR, EAM_1726*), hemin ABC transporter (*hmuV, hmuU, hmuT, hmuS*) (Table 3.2). Those proteins are components of exogenous iron uptake system. Again, most iron-uptake genes exhibited much higher expression in the chemical # 3 treatment than in chemical # 9 treatment. Interestingly, components of iron-sulfur cluster genes (*IscR, sufA, sufC*) were also induced by chemical # 3, suggesting that this compound also interfers with endogenous iron regulation. These

results suggested that salicylidene acylhydrazides commonly activated iron acquisition and utilization in *E. amylovora*.

To validate microarray data, qRT-PCR was carried out for eight genes, including *hrpA, dspE, hrpL*, *hrpN*, *avrRpt2*, *amsG*, *amsD*, *glgB*, *foxR* and *EAM_3350.* These results were consistent with our microarray data (Fig. 3.2). Besides, qRT-PCR analysis revealed that *hrpA* was downregulated by 0.13 and 0.66 fold by chemicals # 3 and # 9, respectively. *hrpN* was downregulated by 0.07 and 0.29 fold by the two compounds, respectively. We reasoned that the discrepancy between the array data and qRT-PCR data is due to the satuation of both genes in the microarray assay (Fig. 3.2).Furthermore, real-time quantification assay is based PCR production of amplification cycling, displaying high sensitivity and accuracy over microarray data (Draghici *et al*., 2005).

qRT-PCR analysis was also performed to determine whether salicylidene acylhydrazides caused a similar transcriptional perturbation in *E. amylovora* . Four *hrp* genes (*hrpA*, *hrpN*, *hrpL* and *dspE*), one amylovoran biosynthesis genes (*amsG*) and two iron uptake genes (*EAM_359 and humS*) were selected. qRT-PCR results showed that salicylidene acylhydrazides consistently suppressed T3SS and *ams* genes while activated iron acquisition genes (Fig. 3.3). For example, *hrpL* expressed at levels 0.51-, 0.20-, 0.24-, 0.7- and 0.19- fold lower, respectively, in the treatment of chemical # 1, # 2, # 3, # 4 and # 9 than in DMSO treatment. In contrary, hemin transport protein encoding gene *hmuS* expressed at levels 52.27-, 5.09-, 50.12-, 9.99- and 22.91- fold higher, respectively, in the treatment of chemical # 1, # 2, # 3, # 4 and # 9 than that of DMSO treatment. Thus, suppressing T3SS or *ams* gene expressions and promoting iron uptake gene expressions may represent a common transcriptional pattern among these chemicals.

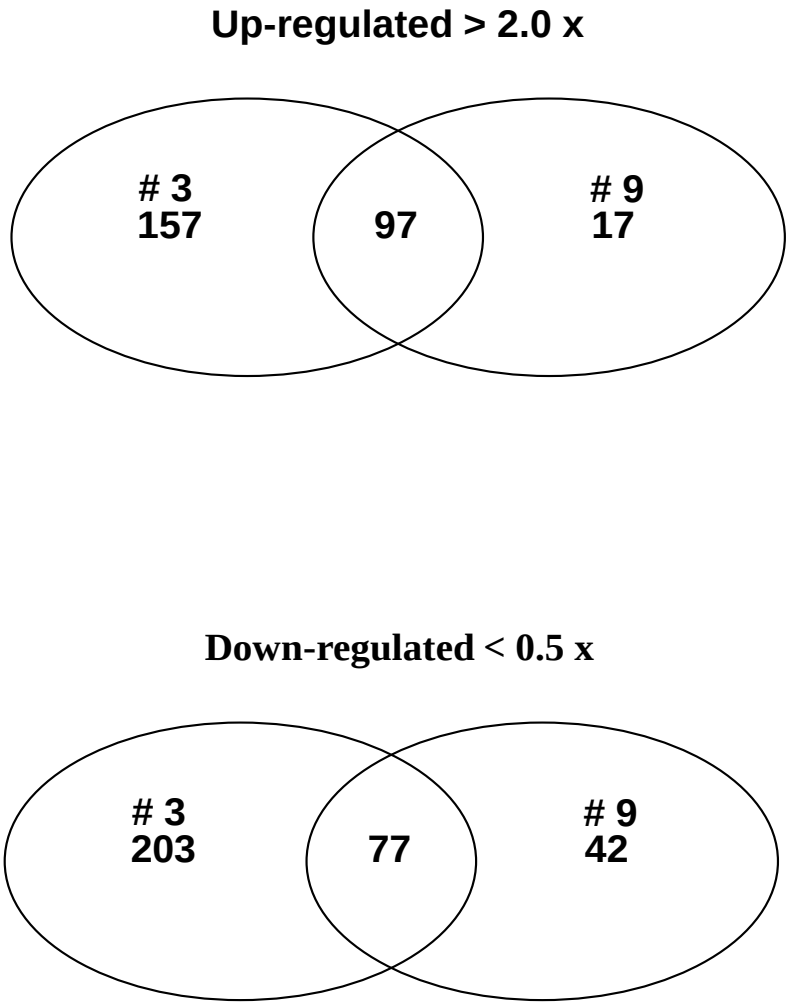


**Fig. 3.1 Venn diagrams displaying the number of differentially expressed genes in chemical treatments compared with DMSO control.** Differential expressed genes are those that are up-regulated (> twofolds) and suppressed (< twofolds) by chemicals # 3 (50 μmol) and # 9 (50 μmol) treatments, respectively, at 18 h when *E. amylovora* Ea273 was grow in HMM at 18 ℃.

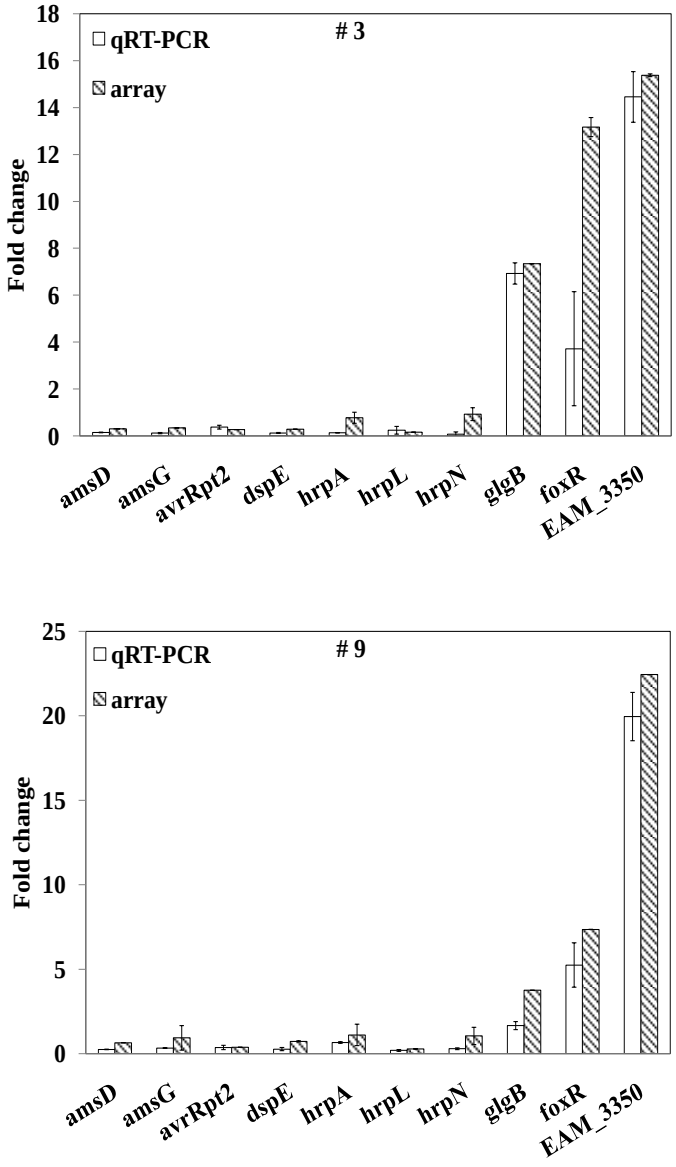


**Fig. 3.2 Verification of microarray gene expression data by qRT-PCR**. The relative fold change of each gene was derived from comparison of either # 3 or # 9 treatment versus DMSO control in HMM. 16s sRNA gene was used as endogenous controls in qRT-PCR. The values of relative fold change were means of three replicates.

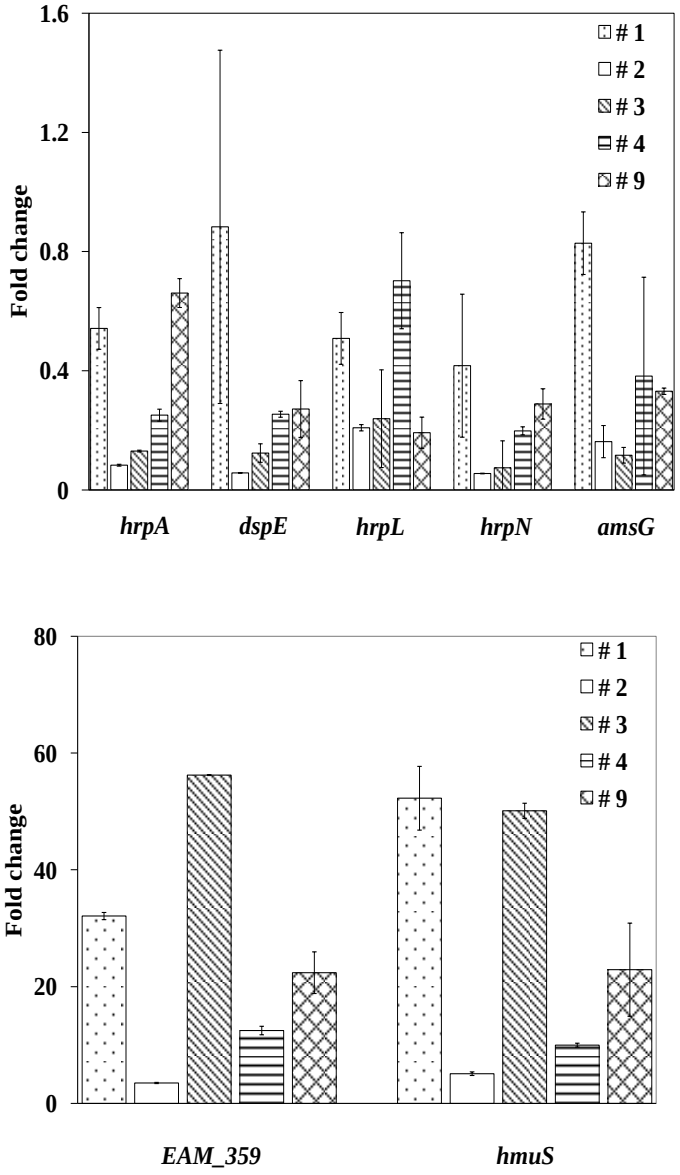


**Fig. 3.3 Effect of salicylidene acylhydrazides on virulence gene expression.** Relative fold changes were determined by qPCR for *hrpA*, *dspE*, *hrpL*, *hrpN*, *amsG*, *EAM_359* and *hmus* genes when *E. amylovora* Ea273 was grown in the presence of chemicals # 1-4 and # 9 in HMM for 18 h at 18 ℃. 16s sRNA gene was used as endogenous controls. The values of relative fold change were means of three replicates.

**Table 3.2 Effects of chemical # 3 and # 9 on transcription of T3SS genes**

| Gene ID | Gene | No.3/DMSO[a] | No.9/DMSO[a] | Protein description |
|---|---|---|---|---|
| *EAM_0423* | *avrRpt2* | 0.27 | 0.38 | cysteine protease protein AvrRpt2 |
| *EAM_2697* | *hopC1* | 0.31 | 0.41 | putative type III effector protein |
| *EAM_2780* | *eop2* | 0.23 | 0.52 | type III effector (pectin lyase) |
| *EAM_2871* | *dspF* | 0.27 | 0.89 [b] | putative avirulence protein |
| *EAM_2872* | *dspE* | 0.28 | 0.73 | type III effector protein |
| *EAM_2873* | *hrpW* | 0.37 | 0.70 [b] | putative pectate lyase |
| *EAM_2877* | *hrpN* | 0.92 [b] | 1.04 [b] | harpin |
| *EAM_2878* | *hrpV* | 0.21 | 0.44 | type III secretion system protein |
| *EAM_2879* | *hrpT* | 0.25 | 0.54 [b] | type III secretion system protein |
| *EAM_2880* | *hrcC* | 0.22 | 0.42 | type III secretion system protein |
| *EAM_2881* | *hrpG* | 0.28 | 0.56 | type III secretion system protein |
| *EAM_2882* | *hrpF* | 0.18 | 0.43 | type III secretion system protein |
| *EAM_2883* | *hrpE* | 0.19 | 0.40 | type III secretion system protein |
| *EAM_2884* | *hrpD* | 0.17 | 0.32 | type III secretion system protein |
| *EAM_2885* | *hrcJ* | 0.24 | 0.44 | type III secretion system protein |
| *EAM_2886* | *hrpB* | 0.39 | 0.64 | type III secretion system protein |
| *EAM_2887* | *hrpA* | 0.77 | 1.11 [b] | type III secretion system protein |
| *EAM_2891* | *hrpS* | 0.41 | 0.97 [b] | Sigma 54 enhancer-binding protein |
| *EAM_2892* | *hrpY* | 0.57 | 0.58 | two-component response regulator |
| *EAM_2893* | *hrpX* | 0.57 [b] | 0.49 [b] | two-component sensor kinase |
| *EAM_2894* | *hrpL* | 0.16 | 0.28 | sigma factor HrpL |
| *EAM_2895* | *hrpJ* | 0.19 | 0.37 | type III secretion system protein |
| *EAM_2896* | *hrpI* | 0.18 | 0.37 | type III secretion system protein |
| *EAM_2897* | *hrpQ* | 0.26 | 0.53 | type III secretion system protein |
| *EAM_2898* | *hrcN* | 0.21 | 0.41 | type III secretion system protein |
| *EAM_2899* | *hrpO* | 0.30 | 0.47 | type III secretion system protein |
| *EAM_2900* | *hrpP* | 0.25 | 0.53 [b] | type III secretion system protein |
| *EAM_2901* | *hrcQ* | 0.18 | 0.48 | type III secretion system protein |
| *EAM_2902* | *hrcR* | 0.23 | 0.53 [b] | type III secretion system protein |
| *EAM_2903* | *hrcS* | 0.17 | 0.44 | type III secretion system protein |
| *EAM_2904* | *hrcT* | 0.32 | 0.57 | type III secretion system protein |
| *EAM_2905* | *hrcU* | 0.19 | 0.45 | type III secretion system protein |
| *EAM_2908* | *hsvC* | 0.06 | 0.35 | conserved hypothetical protein |
| *EAM_2909* | *hsvB* | 0.04 | 0.22 | conserved hypothetical protein |
| *EAM_2910* | *hsvA* | 0.06 | 0.30 | putative amidinotransferase |
| *EAM_2911* | *hrpK* | 0.39 | 0.47 | type III secretion system protein |

[a] Expression ratio ≥2.0 indicates genes are up-regulated in chemical treatments and ≤0.5 indicates genes are down-regulated in chemical treatments. [b] P value >0.05; all others with P value <0.05.

**Table 3.3 Other differentially expressed genes for chemicals # 3 and # 9**

| Gene ID | Gene | No.3/DMSO[a] | No.9/DMSO[a] | Protein description |
|---|---|---|---|---|
| Group I | | | | |
| *EAM_2163* | *amsL* | 0.38 | 0.85 [b] | amylovoran biosynthesis protein |
| *EAM_2164* | *amsK* | 0.26 | 0.56 | glycosyltransferase |
| *EAM_2165* | *amsJ* | 0.33 | 0.69 [b] | amylovoran biosynthesis protein |
| *EAM_2166* | *amsF* | 0.45 | 0.98 [b] | amylovoran biosynthesis protein |
| *EAM_2167* | *amsE* | 0.26 | 0.65 | amylovoran glycosyltransferase |
| *EAM_2168* | *amsD* | 0.30 | 0.65 | glycosyltransferase |
| *EAM_2169* | *amsC* | 0.34 | 0.84 [b] | oligosaccharide repeat unit polymerase |
| *EAM_2170* | *amsB* | 0.28 | 0.71 | glycosyltransferase |
| *EAM_2171* | *amsA* | 0.24 | 0.69 [b] | tyrosine-protein kinase |
| *EAM_2172* | *amsI* | 0.24 | 0.68 [b] | protein-tyrosine-phosphatase |
| *EAM_2173* | *amsH* | 0.28 | 0.72 [b] | amylovoran export protein |
| *EAM_2174* | *amsG* | 0.34 | 0.93 [b] | UDP-galactose-lipid carrier transferase |
| Group II | | | | |
| *EAM_3269* | *glgA* | 6.91 | 3.24 | glycogen synthase |
| *EAM_3270* | *glgC* | 7.18 | 4.03 | glucose-1-phosphate adenylyltransferase |
| *EAM_3271* | *glgX* | 5.06 | 2.43 | glycogen debranching enzyme |
| *EAM_3272* | *glgB* | 7.34 | 3.75 | 1,4-alpha-glucan branching enzyme |
| Group III | | | | |
| *EAM_0358* | *foxR* | 13.17 | 7.35 | ferrioxamine TonB-dependent receptor |
| *EAM_0359* | *dfoC* | 60.30 | 43.63 | decarboxylase |
| *EAM_0360* | *dfoA* | 236.45 | 133.50 | siderophore biosynthesis protein |
| *EAM_0361* | *dfoJ* | 214.85 | 120.14 | siderophore biosynthesis |
| *EAM_1639* | *hmuV* | 59.27 | 37.76 | hemin ABC transporter |
| *EAM_1640* | *hmuU* | 64.50 | 46.38 | hemin ABC transporter |
| *EAM_1641* | *hmuT* | 118.68 | 73.26 | hemin ABC transporter |
| *EAM_1642* | *hmuS* | 99.19 | 71.24 | hemin ABC transporter |
| *EAM_1888* | *tonB* | 2.92 | 4.02 | TonB protein |
| *EAM_2955* | *exbD* | 4.53 | 4.44 | biopolymer transport protein |
| *EAM_2956* | *exbB* | 6.20 | 6.13 | biopolymer transport protein |

[a] Expression ratio ≥2.0 indicates genes are up-regulated in chemical treatments and ≤0.5 indicates genes are down-regulated in chemical treatments. [b] P value >0.05; all others with P value <0.05.

### 3.4 Discussion

A previous study has shown that salicylidene acylhydrazide-mediated transcriptional suppression included all T3SS genes located on the enterocyte effacement (LEE) pathogenicity island as well as that of non-LEE effectors in *E. coli* O157: H7 (Tree *et al*., 2009). It also demonstrated that salicylidene acylhydrazides specifically suppress transcript of all genes on T3SS-1 of *S. Typhimurium* (Layton *et al*., 2010). In this study, a total of 534 and 183 genes were significantly regulated by chemcials # 3 and # 9 treatment under *hrp* inducing condition, respectively. The transcriptional profiling in *E. amylovora* was very similar to those reported for *E. coli* O157: H7 and *S. typhimurium* after exposure of salicylidene acylhydrazides. The majority of genes in the *hrp/ hrc* cluster were commonly down-regulated by the two compounds. Chemical # 3 was more potent in suppressing transcription of T3SS genes than chemical # 9. Moreover, qRT-PCR results showed that several genes were significantly suppressed by small molecules, including *hrpA* and *hrpN*. These results suggest that suppression of T3SS by salicylidene acylhydrazide is a conserved transcriptional response among different bacteria species. HrpL is an ECF subfamily sigma factor that serves as the master regulator of the T3SS of *E. amylovora* (Wei and Beer, 1995). Consistent with previous GFP-promoter activity assay, it was found that *hrpL* was significantly down-regulated about 5 folds by chemicals # 3 and # 9. It is, therefore, likely that reduced transcripts in master regulator HrpL is the mechanism of transcriptional silencing of T3SS in *E. amylovora*.

Moreover, the transcriptional repression of chemical # 3 extended to all amylovoran biosynthesis genes, while # 9 only slightly reduced expression of some *ams* genes. This is the first report of salicylidene acylhydrazide interfering with a different virulence factor. It should be pointed out that salicylanilides were first identified as inhibitors of two component signal transduction system in different bacteria (Macielag *et al*., 1998). Our previous results indicated that the RcsBCD phosphorelay system played an essential role in controlling amylovoran production and partially influenced the expression of T3SS genes (Wang *et al*., 2012). Thus, it is reasonable to believe that salicylidene acylhydrazides might alter the phosphorylation process of RcsBCD system, resulting in interference of signal transduction that regulates amylovoran production.

Another unique feature of salicylidene acylhydrazide-induced transcriptome was the activation of iron uptake system. Layton *et al*. (2010) observed that about one quarter of genes in *S. Typhimurium* significantly regulated after INP0403 treatment are genes involving iron acquisition. These included TonB and its dependent receptors (*exbBD*), ABC ferric transporter (*fepABCDEG*), iron transport proteins (*feoAB, fhuABCDEF*), iron uptake regulator (*fur*) and other iron related genes. Our microarray results recomfirmed this finding and showed that most significantly upregulated genes in *E. amylovora* transcriptome were genes encoding iron uptake proteins, including siderophore DFO biosynthesis to hemin ABC transporter. Furthermore, chemical # 3 also induced genes that involve in endogenous iron-sulfur cluster regulation (*IscR, sufA, sufC*). On the other hand, the two compounds resulted in a reduced transcript of *ftnA* by about 5 folds (data not shown), which is an intracellular iron storage protein. However, although the iron uptake genes are induced in response to these compounds, it is unclear whether bacteria cells indeed could obtain more iron from environment.

Recent studies using genome wide expression profiling indicate that upregulation of iron acquisition system is a characteristic feature of reactive oxygen species (ROS) production during antibiotics-induced cell stress. It is clear that ferrous iron is released from Fe–S clusters when superoxide production is induced by traditional bactericidal antibiotics and disrupts iron regulatory dynamics. Recruitment of "free" ferrous iron is found to generate destructive hydroxyl radicals through Fention chemistry, which subsequently contribute to cell death. Superoxide and hydroxyl radical are important agents of ROS, which is a family of molecules that are continuously generated, transformed and consumed in all living organisms (Dickinson and Chang, 2011). Thus, increasing iron uptake promotes the formation of ROS when bacteria respond to antibiotic stress. Traditional perspective dominantly regards ROS as antimicrobial agents that are able to exert oxidative stress and damage against a wide range of pathogens (Fang, 2011). However, there is also growing recognition that ROS participates in many other biological processes that go beyond simple detriment effect of bacterial killing. Recent studies suggest that ROS can regulate structure and function of a variety of biomolecules by altering their oxidation-reduction (redox) states. In this study, several genes encoding redox proteins were differentially expressed after chemical treatment,

such as *fpr*, *osmC* and *trxC*. It is possible that salicylidene acylhydrazides disrupt iron regulatory dynamics, leading to ROS formation, which then mediates the redox modification of a wide range of biomolecules.

Despite many genes encoding iron uptake and redox regulation were significantly differentially expressed, activation of SOS or oxidative stress response were not observed. Thus, it is likely that under exposure to these small molecules, breakdown of iron regulation causes ROS generation that acts as redox signals, thus disrupting T3SS function and amylovoran biosynthesis. This perspective is in agreement with a recent report that peroxidase Tpx and oxidoreductase WrbA were identified as putative targets of salicylidene acylhydrazides (Wang *et al*., 2011). Interestingly, it was also found that conformational changes of Tpx between the reduced and oxidized states affect its binding ability to salicylidene acylhydrazides (Gabrielsen *et al.*, 2012). Future analysis is needed to address whether treatment of salicylidene acylhydrazides indeed causes ROS formation and what specific ROS is responsible for virulence inhibition. This knowledge may illustrate unknown role of ROS in regulating virulence in bacteria, which may result in new class of antibiotics for fire blight management.

# CHAPTER 4

# SMALL MOLECULE INHIBITORS BLOCKED SECRETION OF T3SS PROTEINS


## Abstract

Small molecule inhibitors provide a new direction for development of new antimicrobial compounds that might disarm rather than kill bacteria. In this study, salicylidene acylhydrazide compounds # 3 and # 9 were identified which can significantly inhibit promoter activities of T3SS genes *in vitro*. Microarray analysis also illustrated that the majority of genes in *E. amylovora* T3SS pathogenicity islands including *hrpL* as well as several effectors including *avrRpt2* and *hopC1* were down-regulated by the two compounds. To test whether salicylidene acylhydrazides affect secretion of T3SS proteins, total secreted proteins from wild type bacteria grown in the presence or absence of chemicals # 3 or # 9 were quantified. Our results showed that chemical # 9 exhibited a dose-dependent inhibition of protein secretion in *E. amylovora*; while chemical # 3 was more effective, causing a complete blockage of secretion of T3SS proteins at as low as 5 μmol.


## 4.1 Introduction

Bacterial virulence factors are required for disease establishment, which could be exploited as ideal target for antibiotic drug discovery. It is reasonable to assume that a weak selection pressure imposed by those compounds upon bacterial population would diminish or slow down resistance development. A number of small molecules were found that targeted different virulence factors, including global regulator, adhesion and invasion protein, quorum sensing, two-component regulatory system and so on (Alksne and Projan, 2000; Keyser *et al*., 2008).

In *E. amylovora*, T3SS is encoded by the *hrp* gene cluster, which is required to elicit hypersensitive response on non-host plant and cause disease on susceptible host plants. T3SS of *E. amylovora* contains 27 genes, encoding two harpin proteins (HrpN and HrpW), several effectors, four regulatory proteins (HrpL, HrpS and HrpXY) and other structural component proteins. Early study demonstrated that *E. amylovora* secretes 12

proteins via T3SS, including DspA/E, HrpK, Eop2, HrpW, Eop1, HrpN, HrpJ, TraF, Eop3, FlgL and HrpA (Nissinen *et al*., 2007). Harpin HrpN is a 40 kDa protein that plays an essential role in virulence and avirulence activities of *E. amylovora* (Wei *et al.*,1992; Boureau *et al*., 2011). Nissinen *et al*. (2007) showed protein band presenting HrpN was weakly detected in the sample of *hrpJ* mutant, suggesting HrpJ was essential for HrpN's extracellular stability. Recent evidence suggests that HrpN might facilitate the translocation process of its own and other effectors when *E. amylovora* deliver a suite of effector proteins (Boureau *et al*., 2011). HrpW (60kDa) contains a pectate-lyase domain homologous with type 3 pectatelyase, and partially participates in callose deposition. *DspA/E*, homologous with *avrE* of *P. syringae* pv. *tomato*, encodes a virulence factor of *E. amylovora* (Bogdanove *et al*.,1998; Gaudriault *et al*. 1997). Gaudriault *et al.* (1997) expressed *dsp* region by using T7 RNA polymerase promoter expression system. Autoradiography visualization revealed that DspE and DspF is a 190 kDa and 15.5 kDa polypeptide, respectively. Bocsanczy *et al.* (2008) reported that protein DspE is translocated into cells of *Nicotiana tabacum*. It was revealed that HrpN and HrpJ are required for DspE translocation into plant cell but not for its secretion from bacterial cell. The precise functions of remaining secreted proteins in *E. amylovora* were unclear. For example, HrpK (80kDa) shows similarity with a putative translocator, while Eop2 (68kDa) has a C-terminal pectate lyase domain but can't be translocated in *P.syringae* pv. *Tomato* (Petnicki-Ocwieja *et al*., 2005; Smits *et al*., 2009). Bands of DspE, HrpK, Eop2, HrpW, Eop1, HrpN, HrpJ, TraF, Eop3, FlgL and HrpA were undetectable in the secretome of mutant strain lacking *hrpL*, suggesting these proteins are secreted in T3SS-dependent manner (Nissinen *et al*., 2007).

Recently, a number of salicylidene acylhydrazides that inhibit T3SS activity have been described. This group of chemicals is known to be broadly effective against different bacteria, including *Yersinia*, *Chlamydiae*, enterohemorrhagic *E. coli*, *Salmonella* and *Shigella* (Kauppi *et al*., 2003 and 2007; Dahlgren *et al*., 2007; Muschiol *et al*., 2006 and 2009; Wolf *et al*., 2006; Veenendaal *et al*., 2009). However, the mode of action of this group compounds remains unknown. It was found that salicylidene acylhydrazides had the ability to suppress expression of T3SS genes in *E. coli* O157: H7 and *S. Typhimurium*, indicating that this group compounds would directly target transcription

factors of T3SS (Tree *et al*., 2009; Layton *et al*., 2010). Further experiments showed that INP0007 and INP0403 inhibited the secretion of *S.* Typhimurium T3SS-1 effector proteins in a dose-dependent manner. The reduced T3SSeffectors resulted in a decrease in the magnitude of the secretory and inflammatory responses when the bacteria infected HeLa cells (Hudson *et al*., 2007). Interestingly, blockage of effector secretion was associated with impaired needle complex in *S. flexneri*, suggesting that these compounds were able to interfere with needle formation or assembly.

In prior screening, salicylidene acylhydrazide compounds# 3 and # 9 were the most effective agents in inhibiting T3SS genes *in vitro*. Our microarray analysis also illustrated that majority of genes of *hrp* cluster of *E. amylovora* including master regulator *hrpL* as well as several effectors including *avrRpt2* and *hopC1* were significantly down-regulated by both compounds. Besides, compound # 3 treatment slightly suppressed the symptom development when *E. amylovora* infected crab apple blossom. It is, therefore, reasonable to hypothesize that these salicylidene acylhydrazides could block the secretion or translocation of T3SS effectors. To test this hypothesis, total secreted proteins from wild type bacteria grown in the presence or absence of selected compounds were quantified.

**4.2 Materials and methods**

Wild type Ea273 was grown overnight in LB broth at 28 ℃. After washing twice by PBS, bacterial cells were re-inoculated in 25 ml of HMM. Chemicals were added to the bacterial suspension to a final concentration of 50, 25, 10, and 5 μmol. These suspensions were incubated at 18 ℃ for 18 h with shaking at 250 rpm. Cells were harvested by centrifugations at 4000 g for 10 min twice, and cell-free supernatants were prepared by filtration using 0.2 um filter (Fisher, Hudson, NH, U.S.A.). Trichloroacetic acid (TCA) was added into resulting supernatants to yield a final concentration of 10 % (w/v). Samples were incubated overnight at −20 °C, and for each sample precipitated proteins were pelleted by centrifugation at 20,000 g at 4 ℃ for 20 min. Bacterial cells were washed by PBS, diluted 10 folds, and then suspended into Laemmli sample buffer(Bio-Rad, Hercules, CA, U.S.A.). Proteins were separated by SDS-PAGE,

visualized by staining with Coomassie Blue R and scanned with an Odyssey infrared imaging system (Li-Cor, Lincoln, NE, U.S.A.).

### 4.3 Results

To characterize effect of small molecules on T3SS-secreted proteins, the secretome from *E. amylovora* was analyzed containing chemicals # 3 or # 9 at different concentrations. At concentrations from 0 to 50 μM, cellular protein amount of chemical treatment was similar to that of DMSO treatment, suggesting the bacterial whole-cell protein remained unaffected by the two compounds. Protein profiling analysis showed that the amount of secreted proteins of *E. amylovora* were reduced after chemical # 9 exposure, which was in a concentration-dependent manner. At the concentration of 25 μmol, no protein on the gel was visible (Fig. 4.1). In contrast, chemical # 3 appeared to be more effective, causing a complete blockage of T3SS protein secretion at the concentration of 5 μmol (Fig. 4.1). These results support our previous findings that the two chemicals suppressed expression of majority genes encoding T3SS, and further suggest that salicylidene acylhydrazides may target mater regulator HrpL or other upstream signal pathway.

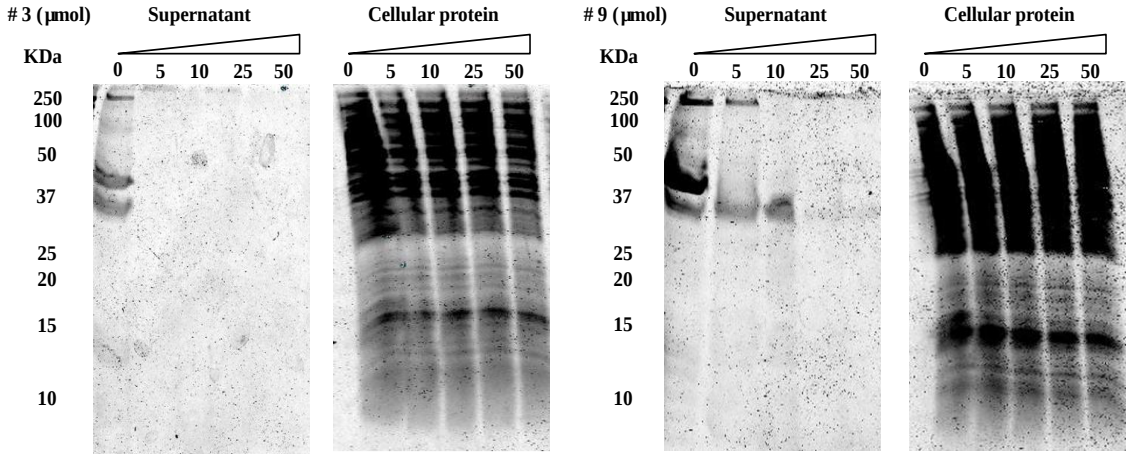


**Fig. 4.1 Effect of small molecules on the secretion of T3SS-related proteins.** *E. amylovora* Ea273 was grown in the presence of different concentrations of chemicals # 3 and # 9 in HMM for 18 h at 18 ℃. Proteins were harvested from supernatants and concentrated by 10 % (w/v) trichloroacetic acid. Proteins were subjected to SDS-polyacrylamide gel electrophoresis (12%) and visualized by staining with Coomassie Blue R.

### 4.4 Discussion

Salicylidene acylhydrazide is the largest family of T3SS inhibitor identified so far (Wang *et al.*, 2011). It was first found to block effector secretion in *Yersinia*. Later evidence suggest that this group of compounds target a broad range of pathogens carrying T3SS, such as *Chlamydiae,* enterohemorrhagic *E. coli*, *Salmonella* and *Shigella* (Kauppi *et al*., 2003 and 2007; Dahlgren *et al*., 2007; Muschiol *et al*., 2006 and 2009; Wolf *et al*., 2006; Veenendaal *et al*., 2009).These studies have demonstrated salicylidene acylhydrazide specifically interfere with T3SS function in many aspects, including gene transcription, effector secretion or translocation, needle assembly and bacterial intracellular replication. Delivery of effector protein from bacterial cytoplasm into plant cell is the prerequisite for *E. amylovora* to manipulate host immune defense. This process is achieved by the T3SS (Cornelis, 2006).The secretion conduit pilus of *E. amylovora* is composed of multiple copies of a single protein called HrpA. Early studies have demonstrated that mutant strain impairing effector secretion was unable to cause disease

symptom and survive in immature pear fruits, in which phenotype is similar to the mutant deleting the T3SS pathogenicity island(Zhao *et al*., 2005; Nissinen *et al*., 2007).

In this study, our results showed that chemicals # 3 and # 9 blocked the secretion of all effector proteins. By comparing the secretome of Ea273 in the early study (Nissinen *et al*., 2007), bands of proteins representing DspE, HrpW, HrpN and HrpJ were absent after chemical treatments. Further mass spectrometry and *de novo* sequencing are needed to determine each band of untreated sample that is detectable on the gel. It is consistent with the result that both chemicals significantly suppressed the transcription of *hrpL*, a master regulator of T3SS in *E. amylovora*. Again, chemical # 3 exhibited much strong capability in reducing secretion of T3SS protein compared to chemical # 9. This finding was comparable to previous results that disease symptom and bacterial growth in crab apple flower was slightly reduced only after chemical # 3 treatment. Combined with the previous studies, we propose that salicylidene acylhydrazide inhibit T3SS gene expression and blocks protein secretion by affecting transcription of the master regulator HrpL. It is also possible that the blockage of T3SS protein secretion by salicylidene acylhydrazides is due to disruption of needle complex assembly.

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